# The teratogenic effects of thalidomide on limbs

## ABSTRACT

Thalidomide remains notorious as a result of the damage it caused to children born to mothers who used it to treat morning sickness between 1957 and 1961. The re-emergence of the drug to treat a range of conditions including erythema nodosum leprosum (a complication of leprosy) has led to a new generation of thalidomide damaged children being born in Brazil. Although thalidomide affects most of the developing tissues and organs of the body, the damage to the limbs is striking. Indeed phocomelia, the severe reduction or loss of the proximal long bones with retention of the distal hand/foot plate remains the stereotypical image of thalidomide. This review focuses on the type and range of damage thalidomide caused to the limbs, reviews current understanding of the mechanisms underlying thalidomide-induced limb malformations and outlines some of the challenges remaining in elucidating its teratogenicity.

Level of evidence criteria: Not applicable as this is a scientific review, could also be classified as Level V.

## THALIDOMIDE – A BRIEF HISTORY

Thalidomide was marketed in 1957 by Chemie-Grunenthal as a non-addictive, non-toxic, non-barbiturate sedative. It was very popular at the time, being distributed in at least 46 countries worldwide as an effective drug in relieving morning sickness. Despite its effectiveness, thalidomide use was associated with peripheral neuropathy in patients and for this reason, Frances Kelsey, a reviewer for the United States Food and Drug Administration (US FDA) refused to licence its distribution in the US (Vargesson, 2015). Subsequently, there were growing suggestions around 1960 that thalidomide use was the cause of an epidemic of severe birth defects in the UK, Europe, Canada, Japan and Australia. By 1961, the link was undeniable which led to the drug being removed from the market at the end of 1961 (Lenz and Knapp, 1962; McBride, 1961).

The type of birth defects seen were severe and striking. Damage to multiple tissues and organs was seen including the eyes, ears, genitals, internal organs including gastrointestinal tract, facial nerves, heart and cardiovascular system as well as limbs (Kajii et al., 1973; Lenz and Knapp, 1962; McCredie and Willert, 1999; Smithells and Newman, 1992; Tajima et al., 2016).

Thalidomide embryonic toxicity occurs in a short time window – now known as the time-sensitive window (day 20-36 post-conception) (Lenz, 1968; Vargesson, 2015). Several studies have since demonstrated that earlier embryonic exposure within this window causes more damage to multiple organ systems (Vargesson, 2013; Vargesson, 2015). It was reported that just a single 50mg tablet was enough to

damage the developing embryo/fetus and that 50% of pregnancies exposed to thalidomide resulted in affected children (Smithells and Newman, 1992).

## THALIDOMIDE – TODAY

At present, it is known that thalidomide has multiple actions in the body, possessing anti-angiogenic, anti-inflammatory and immunomodulatory actions and is now used successfully to treat a wide range of clinical conditions including some cancers, multiple myeloma, erythema nodosum leprosum (ENL) (Franks et al., 2004; Vargesson, 2013; Vargesson, 2015). However, long term clinical use of thalidomide in the adult patient can cause the nasty side-effect, peripheral neuropathy (Vargesson, 2015). Furthermore, and tragically, a new generation of thalidomide survivors has also been seen in Brazil since 1996, which is due to the use of thalidomide to treat ENL and the medicine sharing culture that occurs in Brazil (Castilla et al., 1996; Vianna et al., 2011). This underlines how important it is to understand the complete mechanisms of action of thalidomide to try and make forms that retain clinical benefits without the side-effects of peripheral neuropathy and thalidomide-induced embryopathy.

## THALIDOMIDE-INDUCED LIMB DIFFERENCES

Among the multiple effects of thalidomide, the most striking upon the embryo was to the limbs. Upper limb deficiencies were more common and presentation ranged from triphalangeal thumb, to radial dysplasia (complete loss of radius and thumb and sometimes index finger), to phocomelia (severe shortening and/or loss of the proximal long bones whilst retaining parts of the distal hand/foot plate structures) and even amelia (Lenz and Knapp, 1962; McCredie and Willert, 1999; Newman,

1985; Newman, 1986; Smithells and Newman, 1992; Tajima et al., 2016). Multiple clinical studies have ascertained that some limb elements are more sensitive to thalidomide than others and an order of limb element loss can be determined, for example, the thumb is the most sensitive, followed by the radius, then the humerus, the ulna and finally fingers on the ulnar side (middle, ring and small) (Kajii et al., 1973; Lenz and Knapp, 1962; McCredie and Willert, 1999; Newman, 1985; Newman, 1986; Smithells and Newman, 1992). In the lower limb, when differences were present, the femur is the most commonly affected bone whereas the fibula the least affected bone (Lenz and Knapp, 1962; Smithells and Newman, 1992). Talipes was also seen in some thalidomide survivors and sometimes was the only damage to the lower limb (Smithells and Newman, 1992). In contrast to the upper limb, polydactyly of the toes was sometimes observed, usually presenting as duplication of the great toe (Smithells and Newman, 1992). The seemingly dual ability of thalidomide to cause both limb reduction and digit duplication within the same limb remains a mystery.

Characteristic damage to the shoulder joint and pelvis is also observed in thalidomide embryopathy. Indeed, the acromioclavicular joint of the shoulder is much more prominent and ‘sharp’ in appearance (Smithells and Newman, 1992). The hip joint can be hypoplastic, or in some cases absent (Smithells and Newman, 1992).

Limb differences are usually bilateral, although differences in the left limb is usually more severe than the right (Newman, 1985; Newman, 1986; Smithells and Newman, 1992) and it remains unknown why one side would be more damaged than the other. Indeed, there are some reports of thalidomide exposed survivors with unilateral limb

anomalies, but these occurrences remain rare (Lenz and Knapp, 1962; Schmidt and Salzano, 1980).

## NORMAL LIMB DEVELOPMENT

An understanding of normal embryonic limb development is needed in order to elucidate how thalidomide can cause limb differences. The upper limbs of the developing human embryo begin to form at day 26 post-fertilisation with the lower limbs following 1-2 days later (Vargesson and Hootnick, 2017). The limbs are first seen as buds or protrusions from the flank of the embryo and under the control of several signalling regions, the limb buds then grow out from the embryo body rapidly (Tabin and Wolpert, 2007; Vargesson, 2003). The major signalling centres controlling this outgrowth and subsequent patterning of all bony elements as well as support tissues are the Zone of Polarizing Activity (ZPA) and the Apical Ectodermal Ridge (AER) (Davey et al., 2018; Rodriguez-Niedenfuhr et al., 2001; Vargesson, 2003). The ZPA situated in the posterior mesenchyme of the limb bud controls the anterior-posterior patterning of the limb, for example the formation and identification of the thumb to the small finger in the handplate and the radius and ulna in the forearm (Davey et al., 2018; Tabin and Wolpert, 2007; Tao et al., 2017; Vargesson, 2003). The AER is involved in controlling and regulating proximal-distal outgrowth via the proliferation of cells in the limb bud. The ZPA and AER signal to and through each other to maintain outgrowth and patterning of the various tissues and elements. For example, the ZPA secretes Sonic hedgehog protein and the AER secretes fibroblast growth factor 8 which then signal to each other in a feedback loop, maintaining the respective pathways and at the same time, regulating the expression of many other genes involved in limb patterning (Tabin and Wolpert, 2007; Tao et al.,

2017; Vargesson, 2003). The limb forms and differentiates in a time-dependent proximal to distal fashion, that is, the humerus/femur form before the radius/fibula and ulna/tibia which form before the digits (Davey et al., 2018; Tabin and Wolpert, 2007; Tao et al., 2017; Vargesson, 2003. In order for limb development to occur normally, a variety of other tissues and processes are required, including a rapidly changing vasculature to permit the cell turnover and limb outgrowth as well as programmed cell death to shape the limb elements (Davey et al., 2018; Vargesson, 2003). Precursors of the nerves and muscles enter the limb bud quite late in limb development, long after limb outgrowth has occurred (Mahony et al., 2018). In the human embryo, limbs are fully patterned by day 56 which is then followed by growth and maturation (Rodriguez-Niedenfuhr et al., 2001; Vargesson and Hootnick, 2017) (Figure 1A).

## MECHANISMS OF THALIDOMIDE TERATOGENESIS - MODELS

Many models and theories have been proposed to explain the mechanism underlying the teratogenic actions of thalidomide. In fact, over 30 models have been proposed since the 1960s, including its effects on chrondrogenesis, DNA intercalation, nerve and neural crest damage, vitamin metabolism antagonism and effects on cell adhesion molecules (Stephens et al., 2000; Vargesson, 2013; Vargesson, 2015). While some of these can reasonably explain some of the damages seen in thalidomide survivors there remains continued debate about the precise mechanism of action, that explains all the damage, variability and time sensitive nature of thalidomide embryopathy.

Current thinking favours thalidomide's ability to bind cereblon and disrupt molecular signalling (Donovan et al., 2018; Ito et al., 2010; Matyskiela et al., 2018); inhibit

angiogenesis (D'Amato et al., 1994; Therapontos et al., 2009; Vargesson, 2009) and induce cell death and reactive oxygen species (Hansen and Harris, 2013; Knobloch et al., 2007). There remains the possibility that these three mechanisms are not mutually exclusive and together result in the damage.

**Cereblon (CRBN) Model**

CRBN is a direct binding partner of thalidomide (Ito et al., 2010). As a ubiquitin ligase, CRBN forms a complex to tag other signalling molecules for destruction. CRBN was identified as a target of thalidomide in a biochemical binding assay and when the protein was mutated to prevent thalidomide association, chicken and zebrafish embryos were unharmed following thalidomide exposure (Ito et al., 2010). However, precisely how CRBN binding to thalidomide results in thalidomide-induced limb damage and the variability between individuals in humans is still currently unexplained, although recent work is beginning to shed some light on this (Donovan et al., 2018; Matyskiela et al., 2018).

Since the discovery of CRBN, a great deal of research has been focused on its role in mediating the role of thalidomide in adult conditions like myeloma (Ito and Handa, 2016; Vargesson, 2015). This has led to the discovery that CRBN needs to recruit additional factors to the CRBN-thalidomide complex before resulting in specific actions. For example, in the treatment of myeloma, thalidomide binds CRBN which then recruits downstream proteins (IKAROS and AIOLOS) to successfully target myeloma cells (Chamberlain et al., 2014; Fischer et al., 2014; Ito and Handa, 2016).

Similarly, recent work demonstrates that following thalidomide binding, CRBN needs to target (and repress) another factor with important roles in embryonic development, namely SALL4, which is a transcription factor of the Spalt-like family (Donovan et al., 2018; Matyskiela et al., 2018). The discovery of an interaction of thalidomide and cereblon with SALL4 is exciting because mutations in SALL4 are known to result in the following human conditions: Duane-Radial ray syndrome (DRRS), also known as Okihiro syndrome (Kohlhase and Holmes, 2004; Kohlhase et al., 2003); Acro-Renal-Ocular syndrome (AROS) (Kohlhase et al., 2005) and the very rare IVIC syndrome, also known as Oculo-oto-radial syndrome (Paradisi and Arias, 2007). Together these conditions share many striking similarities with thalidomide embryopathy (TE) and indeed have been confused for TE previously and have also been termed thalidomide phenocopies (Kohlhase and Holmes, 2004; Kohlhase et al., 2003). These patients can present with a short humerus/radius/thumb deficiency, laterality differences where the reduction deficit differs between left and right limbs, as well as anomalies to the ears, eyes and internal organs. However, unlike TE, the legs are usually unaffected in these syndromes (Kohlhase et al., 2005; Kohlhase and Holmes, 2004; Kohlhase et al., 2003; Smithells and Newman, 1992).

Additional evidence supporting a role for CRBN in TE comes from studies demonstrating that the teratogenic enantiomer of thalidomide binds CRBN but the sedative enantiomer does not (Mori et al., 2018). Thalidomide has the ability to switch between two enantiomer forms in body fluids (Franks et al., 2004; Vargesson, 2013). There are also species differences in CRBN activity, such that thalidomide-sensitive species can bind CRBN and degrade substrates, whereas mice and rats which are thalidomide-insensitive though possessing CRBN have structural changes

194 resulting in the failure of degradation (Kronke et al., 2015). Moreover, the ability of
195 the thalidomide/cereblon complex to bind to and inhibit SALL4 is also species-
196 specific and only occurs in thalidomide-sensitive species (Donovan et al., 2018;
197 Matyskiela et al., 2018). This could explain the famous species-specific effects of the
198 drug, where mouse and rat embryos are insensitive to the teratogenic actions of the
199 drug (Vargesson, 2013; Vargesson, 2015).
200
201 The discovery of SALL4 as a target of the thalidomide-cereblon complex is exciting,
202 and in many respects, makes sense, given the similarity in upper limb differences
203 and some other tissue malformations between thalidomide embryopathy and DRRS,
204 AROS and IVC. Moreover, SALL4 has been previously been proposed to be a target
205 of thalidomide (Knobloch and Ruther, 2008; Kohlhase et al., 2003; Kohlhase et al.,
206 2005). However, whether this molecular interaction with thalidomide causes all the
207 tissue malformations and changes seen in thalidomide embryopathy is yet to be fully
208 demonstrated. In addition, just how this molecular interaction results in the actual
209 tissue malformations is also unclear. Furthermore, DRRS, AROS and IVIC affects
210 the upper limbs and not usually the lower limbs, which can also be affected in TE
211 (Kohlhase et al., 2005; Kohlhase and Holmes, 2004; Kohlhase et al., 2003; Smithells
212 and Newman, 1992). This suggests that there may be other targets of CRBN,
213 perhaps tissue specific or indeed there may be other binding partners or actions of
214 thalidomide.
215
216 Indeed, many other molecular targets of thalidomide have been proposed previously
217 (Vargesson, 2015); for example, several genetic screens in embryonic stem cells
218 and animal (non-human primate) embryos following thalidomide exposure show

thousands of gene expression profile changes, and CRBN is not identified in many of these (Ema et al., 2010; Meganathan et al., 2012).

**Anti-angiogenesis Model**

A separate (but likely inter-related) theory is through the ability of thalidomide to prevent and destroy blood vessel formation (anti-angiogenesis) (Vargesson, 2013; Vargesson, 2015). Thalidomide was first shown to be anti-angiogenic in rabbit and rodent cornea assays in the 1990's (D'Amato et al., 1994; Kenyon et al., 1997). Yet, experimental evidence from chicken embryos suggested effects on limb vasculature several decades earlier (Jurand, 1966). Since these findings, the anti-angiogenic roles of thalidomide have been used in adults successfully to treat conditions including hereditary hemorrhagic telangiectasia (HHT), diabetic retinopathy and some cancers, through the prevention of vessel leakage and also by suppressing angiogenesis (Lebrin et al., 2010).

Such a mechanism has been experimentally demonstrated to explain the range, occurrence of damage, the timing as well as the global nature of the drugs action. For example, an anti-angiogenic analogue of thalidomide, called CPS49, was shown to be able to induce, in a time-sensitive manner, a range of damages including phocomelia-like limbs, radial dysplasia and other anomalies (Davey et al., 2018; Therapontos et al., 2009). CPS49 was demonstrated to affect newly formed or newly forming vessels only (Therapontos et al., 2009). These effects were rapid and occur before changes were seen in expression patterns of important limb development genes and followed by the induction of cell death (Therapontos et al., 2009; Vargesson, 2009). Furthermore, vessel loss and its effects upon the limb occurs

before nerve innervation of the limbs (Mahony et al., 2018). Moreover, nerve inhibition before and during limb outgrowth does not cause thalidomide-like limb damage (Mahony et al., 2018).

Several other studies have demonstrated thalidomide induces vessel loss and/or haemorrhages in embryos (Jurand, 1966; Knobloch et al., 2007; Sorensen et al., 2017; Tamilarasan et al., 2006). We also know that thalidomide survivors themselves exhibit a wide range of cardiovascular changes including heart defects, some of which might actually contribute to the differences seen in thalidomide survivors (Tajima et al., 2016; see also Vargesson and Hootnick, 2017). In further support of this mechanism, multiple anti-angiogenic drugs used as anti-cancer agents, for example sunitinib and sorafenib (Beedie et al., 2016a), which induce cell death, have a wide range of molecular targets and all of these can cause limb damage (Beedie et al., 2016a; Beedie et al., 2017). This indicates that vessel inhibition is a key element underlying drug-induced limb defects.

Blood vessels are essential for cells and tissues to be supplied by nutrients and to remove waste products (Vargesson, 2003). The limbs develop rapidly and undergo major changes almost continuously and as a result require a rapidly changing vasculature to accommodate development, growth and differentiation (Vargesson, 2003; Vargesson and Hootnick, 2017). Around the fifth to sixth week of human embryonic development, cartilage condensations begin forming in the limbs, with the proximal elements condensing before the more distal element and as they do the local vessels must regress (Rodriguez-Niedenfuhr et al., 2001; Vargesson and Hootnick, 2017). The cartilage condensations begin to form bone from the sixth and

seventh weeks, and require vascularization to allow the process to continue through nourishment and to maintain the chrondrified bone (Rodriguez-Niedenfuhr et al., 2001; Vargesson and Hootnick, 2017). Different bony elements have different amounts of vascularization, for example, the ulna has several arterial supplies (ulnar and median artery), yet the radius has one, the radial artery (Vargesson and Hootnick, 2017). Thus, the radius is more sensitive to vascular injury than the ulna and this might explain why in some thalidomide survivors the radius is missing but the ulna remains.

At the time the cartilage condensations are appearing, the vascular pattern in the developing limb undergoes a transition from the embryonic state to the final adult pattern by the end of the seventh week of development (Figure 2; Vargesson and Hootnick, 2017). This has led to suggestions that limb malformations may arise from injury or failure to transit to the adult pattern which normally permits the correct vascularization for forming bony elements and limb formation (Vargesson and Hootnick, 2017). Indeed, there is evidence that limb differences in human foetuses have missing or misplaced vessels (Hoyme et al., 1982; Van Allen et al., 1982; Vargesson and Hootnick, 2017). For example, in limbs with radial dysplasia, the radial artery is malpositioned and has been alleged to be the cause of the bony loss (Hoyme et al., 1982; Van Allen et al., 1982). Finally, Holt-Oram syndrome patients exhibiting limb reduction anomalies (which can be confused with TE) have a significantly reduced peripheral vasculature; indeed, it may be difficult to discern a palpable pulse in such patients (DuPre and Fincher, 1993), a characteristic often demonstrated in the limbs in thalidomide survivors (Smithells and Newman, 1992;

Tajima et al., 2016). Thus, thalidomide-induced inhibition of vessel formation could result in the tissue differences in the limbs.

**Cell death and reactive oxygen species induction model**

Thalidomide has the ability to induce reactive oxygen species in tissues (which can damage and harm tissues) and cell death in embryonic limbs (Hansen and Harris, 2013; Knobloch et al., 2007). When reactive oxygen species are prevented from being pharmacologically induced, embryos are no longer damaged by thalidomide when directly exposed to the drug (Hansen and Harris, 2013). Precisely how reactive oxygen species and cell death is induced by thalidomide in specific tissues and bones and not all cells remains unclear.

In summary, when taken together, the different mechanisms that have been proposed and outlined herein are likely inter-related for the explanation of thalidomide-induced limb differences. The destruction of vessels leading to subsequent localised induction of cell death in tissues can explain the occurrence, range and timing of thalidomide-induced limb defects. However, given thalidomide's species sensitivity, where pregnant rodents embryos are insensitive to thalidomide, this indicates that there must be species-specific mechanisms or molecular target/s, for example, CRBN, which then triggers the cascade of events, including effects on blood vessels and cell death induction, to result in the actual tissue damage.

## CURRENT UNDERSTANDING OF THE MECHANISM OF THALIDOMIDE-INDUCED LIMB TERATOGENESIS

As mentioned, recent work that suggests thalidomide interacts with the molecular target CRBN to repress SALL4 to cause thalidomide phenocopies such as DRRS, AGOS and IVIC in embryos (Donovan et al., 2018; Matyskiela et al., 2018) is exciting, although presently how this interaction results in the actual tissue differences remains unclear. In addition, whether this molecular interaction is responsible for all the tissue malformations induced by thalidomide in the embryo also remains to be determined. Another binding target of CRBN was recently identified, argonaute2 (AGO2) (Xu et al., 2016). This molecule has roles in angiogenesis suggesting that CRBN's interaction with thalidomide could also influence angiogenesis via AGO2, further suggesting CRBN may have multiple downstream targets, and that the precise molecular pathway/s require further elucidation.

Limb differences likely result from downstream events such as vessel loss/inhibition which disrupts the embryonic to adult vascular transition, induction of localised cell death resulting in disruption of normal molecular signalling in the limb and ultimately in tissue loss or further malformation of tissues (Figure 3). As the effect of thalidomide wears off (half-life 8-12hr; Franks et al., 2004), the limb tries to recover and re-establish the normal signalling and morphogenetic events. Condensations of cartilage cells will attempt to form, and depending on the extent of tissue loss may be shorter or may not form at all. They will then be vascularised by surviving vessels, assuming they are present, resulting in smaller limbs and specific bone loss. The damage is then further exacerbated by secondary cell induction changes; nerves and muscle cells migrate into the limb but as the limb tissue is either not there or not

343 in the correct place, nerves and muscles are misplaced, thus exacerbating further
344 damage (Therapontos et al., 2009; Vargesson, 2009) (Figure 1B; Figure 3).
345
346 **THE FUTURE**
347 Understanding precisely how thalidomide causes embryonic malformation could help
348 shed light on how congenital conditions like DRRS, AGOS and IVC come about and
349 is key to understanding if clinically relevant versions of the drug, without the spectre
350 of causing birth defects, can ever be made. This is especially relevant today in
351 countries like Brazil, where the accidental use of thalidomide by pregnant women
352 when treating complications of leprosy has led to a new generation of thalidomide
353 survivors. Encouragingly, some inroads have been made through the production of
354 structural analogs without anti-angiogenic actions (Beedie et al., 2016b).
355
356 With the greatly renewed interest in thalidomide for adult treatments and the revived
357 interest in the teratogenic mechanisms, it must only be a matter of time before we
358 finally uncover the precise mechanism(s) of the drug.
359

## FIGURE LEGENDS

Figure 1 – Limb development and model of how thalidomide affects limb development

A. Normal limb development and outgrowth is regulated by the apical ectodermal ridge (AER) and zone of polarizing activity (ZPA), maintaining outgrowth and gene expression resulting in the final, normal adult pattern.
B. Thalidomide inhibits angiogenesis resulting in cell death and loss of signalling between the AER and ZPA. Prolonged exposure results in amelia (no limb or a rudiment of bone); short exposure results in temporary loss of signalling pathway loss, which recovers to allow remaining cells to be patterned, as these cells are near the AER they will be distal at the expense of proximal cells, resulting in phocomelia.

Figure reproduced with permission from (Vargesson, 2015).

Figure 2 – Vascular transition

During human limb development a transition from the embryonic capillary network to the adult pattern occurs between weeks 5 and 7. Figure reproduced with permission from (Rodriguez-Niedenfuhr et al., 2001).

Figure 3 – Framework of thalidomide induced embryonic damage

Reproduced with modifications and permission from (Vargesson, 2015).

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570