Novel therapeutic strategies targeting telomere maintenance mechanisms in high-risk neuroblastoma

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Abstract

The majority of high-risk neuroblastomas can be divided into three distinct molecular subgroups defined by the presence of MYCN amplification, upstream TERT rearrangements or alternative lengthening of telomeres (ALT). The common defining feature of all three subgroups is altered telomere maintenance; MYCN amplification and upstream TERT rearrangements drive high levels of telomerase expression whereas ALT is a telomerase independent telomere maintenance mechanism. As all three telomere maintenance mechanisms are independently associated with poor outcomes, the development of strategies to selectively target either telomerase expressing or ALT cells holds great promise as a therapeutic approach that is applicable to the majority of children with aggressive disease. Here we summarise the biology of telomere maintenance and the molecular drivers of aggressive neuroblastoma before describing the most promising therapeutic strategies to target both telomerase expressing and ALT cancers. For telomerase-expressing neuroblastoma the most promising targeted agent to date is 6-thio-2′-deoxyguanosine, however clinical development of this agent is required. In osteosarcoma cell lines with ALT, selective sensitivity to ATR inhibition has been reported. However, we present data showing that in fact ALT neuroblastoma cells are more resistant to the clinical ATR inhibitor AZD6738 compared to other neuroblastoma subtypes. More recently a number of additional candidate compounds have been shown to show selectivity for ALT cancers, such as Tetra-Pt (bpy), a compound targeting the telomeric G-quadruplex and pifithrin-α, a putative p53 inhibitor. Further pre-clinical evaluation of these compounds in neuroblastoma models is warranted.

In summary, telomere maintenance targeting strategies offer a significant opportunity to develop effective new therapies, applicable to a large proportion of children with high-risk neuroblastoma. In parallel to clinical development, more pre-clinical research specifically for neuroblastoma is urgently needed, if we are to improve survival for this common poor outcome tumour of childhood.

Keywords: Neuroblastoma, Telomere, Telomerase, MYCN, TERT, Alternative lengthening of telomeres

Background

Neuroblastoma is a common childhood malignancy arising from the sympathetic nervous system. It most commonly arises from the adrenal gland and presents with an abdominal mass, but has a heterogeneous clinical phenotype. Children aged less than 18 months often present with widespread metastases to the skin, liver and bone marrow (stage MS disease) [1], however this can spontaneously resolve without treatment. Conversely, distant metastatic spread in children aged greater than 18 months (stage M disease) is associated with an aggressive clinical phenotype and poor survival [1, 2].

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Children aged >18 months with stage M disease and/or amplification of the MYCN oncogene are classified as having clinical high-risk disease. High-risk neuroblastoma remains a major therapeutic challenge with survival rates of <50% despite intensification of therapy [2, 3]. However, until recently, in the absence of MYCN amplification, the molecular drivers of aggressive disease were unknown.

In 2015 it was reported that aggressive neuroblastoma can be divided into 3 almost mutually exclusive subgroups with either MYCN amplification, rearrangements upstream to the telomerase reverse transcriptase (TERT) gene or alternative lengthening of telomeres (ALT) [4, 5]. Each subgroup is associated with the activation of a telomere maintenance mechanism (TMM) and poor outcomes. Conversely, it is thought that the absence of a TMM is associated with spontaneous regression and excellent survival [6].

Here we summarise TMMs in cancer, specifically focusing on the molecular alterations driving telomere maintenance in neuroblastoma, before describing potential novel therapeutic strategies to directly target TMMs for children with neuroblastoma.

**Biology of telomere maintenance**

Telomeres are regions of repetitive nucleotide sequences (TTAGGG) located at the ends of chromosomes that protect chromosomes from DNA damage, unnecessary DNA repair and fusion with other chromosomes. In normal dividing cells, with each cell replication telomeres gradually shorten, until a critical level is reached, the Hayflick limit, after which cells undergo senescence [7]. This gradual shortening of telomeres associated with cellular aging is believed to be a protective mechanism against uncontrolled growth, preventing cancer development in humans and other mammals [8]. In keeping with this, the activation of a TMM to prevent the shortening of telomeres is necessary for the continued sustained proliferation of cancer cells and hence a hallmark of cancer [9].

Telomerase, a functional ribonucleoprotein enzyme complex, maintains telomere length by adding telomeric DNA repeats at the 3′ ends of linear chromosomes. It is a reverse transcriptase that consists of a catalytic protein subunit TERT, and an essential RNA component known as human telomerase RNA (hTR), encoded by hTERC. hTR acts as a template for the synthesis of telomere DNA and is involved in the catalysis, localisation, and assembly of the telomerase holoenzyme [10, 11]. Telomerase is widely expressed in human embryos between 16 and 20 weeks gestation, however by the early neonatal period telomerase activity can no longer be detected in most somatic tissues [12]. In contrast, the majority of cancers overexpress telomerase: in a systematic analysis of 31 tumour types, over-expression of TERT was identified in 73% of all cancers [13]. This was most commonly associated with genetic alterations in either the TERT gene/promoter or TERT promoter methylation.

ALT is defined as maintenance of telomeres in the absence of telomerase activity [14]. It can be detected in 10–15% cancers overall but is particularly prevalent in tumours of mesenchymal origin [14, 15]. There is a strong association between ALT and loss of function (LoF) genetic alterations in ATRX (Alpha Thalassemia mental Retardation-X linked) in multiple malignancies, including neuroblastoma [13, 16–18].

A number of different non-canonical homologous recombination (HR) based mechanisms have been proposed to play a role in ALT telomere maintenance [19–22]. Furthermore, a number of studies have focused on the underlying basis for the relationship between ATRX LoF and the development of the non-canonical HR mechanisms implicated in ALT (summarised in Fig. 1). Firstly an established role of ATRX is the maintenance of genomic stability via the deposition of H3.3 into telomeric regions [24, 25]. In the absence of ATRX, G4 quadruplex structures may occur in guanine rich regions of DNA such as

![Fig. 1](image-url)
 Telomeres and telomere lengthening in neuroblastoma

Amplification of the MYCN oncogene is found in almost 40% of clinical high-risk neuroblastomas [4, 41], and is associated with up-regulation of TERT expression and telomere dysfunction [4, 42]. In an additional 23–31% of high-risk neuroblastomas, TERT is activated through chromosomal rearrangements involving 5p15.33, proximal to the TERT gene, which induces transcriptional up-regulation of TERT by juxtaposing the TERT coding sequence with strong enhancer elements [4, 5]. The third distinct sub-group, accounting for 24% of high-risk neuroblastoma cases are those with ALT [36]. Approximately half of ALT neuroblastomas are associated with somatic alterations in ATRX [4, 5, 36]. In neuroblastoma, genetic alterations in ATRX are associated with a distinct clinical phenotype including older age at diagnosis and a chronic progressive disease course [43].

Neuroblastoma harbouring a TMM is associated with a poor prognosis regardless of clinical stage [4, 5, 44–46]. More recently it has been shown that in the presence of a TMM a concurrent mutation in a TP53 or RAS pathway associated gene is associated with an even worse prognosis [6]. Conversely, neuroblastoma with TP53 or RAS pathway mutations (including canonical ALK mutations) in the absence of a concurrent TMM are not associated with worse survival and can spontaneously regress [6]. This molecular risk stratification of neuroblastoma, as described by Ackermann et al. [6] is summarised in Fig. 2.

Although the somatic alterations driving TMM's in the majority of neuroblastoma are well defined, high telomerase expression can occur in the absence of either a TERT translocation or MYCN amplification [6]. Also in the absence of an ATRX alteration the underlying drivers of ALT neuroblastoma are currently unknown, and somatic alterations in other genes, known to be associated with high-risk neuroblastoma are currently unknown [4, 36].
with ALT in other malignancies are rarely found in neuroblastoma [47]. Numerous additional mechanisms for telomere maintenance in neuroblastoma have been suggested. A study has identified interstitial telomeric sequences at sites of unbalanced translocations in neuroblastoma cell lines and postulated that these may contribute to a defective telomere maintenance pathway [48]. Another possibility is that genetic predisposition may contribute to the development of TMMs. In keeping with this, six common single nucleotide polymorphisms known to be associated with telomere lengthening have been found to be associated with an increased risk of neuroblastoma [49]. In keeping with the hypothesis that additional underlying predisposing factors may contribute to TMMs, a substantial increase in telomeric DNA damage and active telomere trimming has been described consistently across all high-risk neuroblastomas, regardless of telomerase or ALT status [50].

In summary, data is accumulating to support the hypothesis that TMMs are the common defining molecular feature of aggressive disease in the majority of children with clinical high-risk neuroblastoma. Therefore, both telomerase and ALT represent attractive targets for the development of novel therapeutic strategies with the potential to benefit a significant proportion of high-risk neuroblastoma patients. Here, we review the current pre-clinical and clinical research focused on targeting telomere maintenance with a specific focus on the potential relevance to patients with neuroblastoma.

**Drugs targeting telomerase activity**

**Imetelstat (GRN163L)** is a competitive telomerase inhibitor with a complementary structure to the template region of the RNA component of telomerase that binds to and blocks the active site of the enzyme [51]. Confirmation of target inhibition and pre-clinical efficacy of imetelstat has been demonstrated in multiple cancer subtypes [52–54].

A phase I trial of imetelstat in 20 children with refractory or recurrent solid tumours demonstrated telomerase inhibition, which was sustained through to day 8 of cycle 1 of therapy [55]. Two of 16 patients had a partial response, however no responses were seen in any of the 6 patients with neuroblastoma enrolled on the trial. The main dose limiting toxicities were neutropenia, thrombocytopenia, and lymphopenia. A Phase II study on imetelstat in children with recurrent or refractory central nervous system malignancies was also associated with significant haematological toxicity and was discontinued after two children died of intra-tumoral haemorrhage [35], thus paediatric development for this compound has subsequently been discontinued.

**BIBR1532** is a potent synthetic, non-nucleoside telomerase inhibitor [56]. However like imetelstat there are significant concerns regarding toxicity [57], and this agent has not yet been evaluated in clinical trials.

**Sodium metaarsenite (KML001)** binds to telomeric sequences, displacing hTERT from the nucleus into the cytoplasm [58] and has been shown to be cytotoxic in neuroblastoma cells in vitro [59]. In a phase I trial in adults with advanced solid tumours, objective responses to KML001 and cisplatin were seen in four out of 18 patients, however this trial was also discontinued due to toxicity [60].

**Telomestatin** stabilises G-quadruplexes, which in turn, inhibits telomerase activity [61]. This compound has been shown to induce apoptosis in-vitro in telomerase expressing neuroblastoma cell lines [62] but is not yet in clinical development.

**6-thio-2′-deoxyguanosine (6-thio-dG)** is a nucleoside analogue, which in telomerase active cells is recognised by telomerase and incorporated into telomeres resulting in telomere dysfunction [63]. Pre-clinical efficacy has been demonstrated in melanoma, non-small cell lung cancer and paediatric brain tumour models [64–66]. Although 6-thio-dG has not yet been evaluated in clinical trials, due to the novel mechanism of action of the compound it is thought to be less toxic than traditional telomerase inhibitors [67]. 6-thio-dG has been shown to be effective in-vivo in neuroblastoma models with TERT activation. However, the response in MYCN amplified...
xenografts was mixed, likely reflective of the additional oncogenic pathways activated by MYCN [47]. This is a priority compound for further development for neuroblastoma (Table 1).

**XAV939** is an inhibitor of tankyrase, a positive regulator or telomerase. It has been shown to induce apoptosis in the telomerase expressing neuroblastoma cell line SH-SY5Y [70].

**Drugs targeting ALT**

**Ataxia telangiectasia mutated (ATM) inhibitor combination therapy**

It has been reported by Koneru et al. that ALT neuroblastoma cell lines are more resistant to topoisomerase inhibitors and that activation of ATM at ALT telomeres is associated with chemoresistance. The ATM inhibitor AZD0156 was found to be synergistic with temozolomide and irinotecan therapy in both in vitro and in vivo models of ALT neuroblastoma [71]. AZD0156 is currently in phase I trials in adults [72].

**Ataxia telangiectasia and Rad3 related (ATR) inhibitor**

In 2015 Flynn et al. reported that the presence of ALT renders cells hypersensitive to ATR inhibition in osteosarcoma models [73]. We have evaluated the clinical ATR inhibitor AZD6738 [74], in a panel of neuroblastoma cell lines and found that in contrast, ALT cell lines are relatively resistant to ATR inhibition in comparison to other neuroblastoma cell lines (Fig. 3a-b). This is in keeping with a subsequent publication which directly refuted the findings of Flynn et al. and concluded that differences in ATR inhibitor sensitivity were not related to ALT [77]. Taken together this data does not currently support a role of ATR inhibitors as an ALT specific therapy.

Conversely, we found that MYCN amplified neuroblastoma cell lines show a significantly greater in vitro sensitivity to single agent AZD6738 than non-MYCN amplified neuroblastoma cell lines (Fig. 3a-c), and a preliminary dose finding study demonstrated sensitivity to single agent AZD6738 in the Th-MYCN transgenic mouse model of MYCN driven neuroblastoma [76] (Fig. 3d-e). This is in keeping with other cMYC driven tumours where oncogene driven replicative stress results in a reliance on ATR signalling [78, 79]. Also in keeping with this, the SKNAS neuroblastoma cell line is relatively sensitive to AZD6738 (Fig. 3a) and known to over-express cMYC [80]. In MYCN amplified neuroblastoma models, ATR has also been proposed to play a role in resolution of transcription/replication conflicts [81].

**Pre-clinical research in other ALT cancers**

Recent pre-clinical data focused on the therapeutic targeting of ALT in other cancer subtypes may give important insight into potential therapeutic vulnerabilities that can be exploited for ALT neuroblastoma (Table 1): Tetra-Pt (bpy) is a cisplatin derivative that inhibits telomeric homologous recombination by targeting the telomeric G-quadruplex and has been shown to inhibit growth of ALT-cell xenograft tumours in mice [82]. Thus far this agent has not entered clinical trials nor has been evaluated in neuroblastoma models. CX5461 is an RNA polymerase I inhibitor which has been shown to selectively kill ATRX mutant cells due to its effects on ribosomal RNA transcription. In-vitro sensitivity in ALT cancer cell lines has been demonstrated [83]. It has also recently been reported that some ALT cancer cells rely on p53 and AKT activity to suppress apoptosis. Furthermore the authors go on to show that the p53 inhibitor pifithrin-α suppresses tumour growth in an ALT xenograft model [84] although others have shown that pifithrin-α is not a specific inhibitor of p53 [85], calling into question the underlying mechanism of the demonstrated pifithrin-α response. This strategy may however represent a relevant novel therapeutic opportunity for ALT neuroblastoma, particularly in view of the fact that the majority of neuroblastoma is TP53 wild type [86]. Finally, preliminary data has shown that ALT cancer cell

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**Table 1** Compounds shown pre-clinically to target telomere maintenance mechanisms, to be prioritised for evaluation in neuroblastoma

| Drug          | Target    | Pre-clinical Data                                                                 | Clinical Trials                                      |
|---------------|-----------|----------------------------------------------------------------------------------|------------------------------------------------------|
| 6-thio-dG     | Telomerase| Pre-clinical efficacy in TERT activated neuroblastoma models                     | No                                                   |
| Tetra-Pt (bpy)| ALT       | In-vitro and in-vivo activity in U2OS ALT osteosarcoma model                     | No                                                   |
| CX-5461       | ALT       | In-vitro activity in U2OS and SAOS2 ALT osteosarcoma cell lines                  | On-going adult phase I clinical trial (NCT0271997)   |
| Pifithrin-α   | ALT       | In-vitro and in-vivo activity in U2OS ALT osteosarcoma model                     | No                                                   |
| Trabectedin   | ALT       | In-vitro activity in a panel of ALT cell lines (sarcoma, breast cancer and melanoma) | -FDA approval for certain soft tissue sarcomas [68]   |
|               |           |                                                                                  | -Paediatric sarcoma phase II data [69]               |
|               |           |                                                                                  | -On-going paediatric clinical trials (NCT04067115)   |
lines are in general more sensitive to trabectedin, although the mechanism for this is not clear [87].

Tumour heterogeneity, evolution and TMM targeted therapeutics

In some other paediatric malignancies, ALT (identified by the presence of ALT associated PML bodies) and telomerase activation have been shown to co-exist in the same tumour [88, 89]. In neuroblastoma, intra-tumoural diversity of telomere length in individual tumours has been identified using quantitative telomere fluorescence in-situ hybridisation (FISH) [90], however this is not a sufficiently sensitive or specific biomarker of ALT activity [14, 36]. In fact, one study has identified a subset of neuroblastomas with extremely long telomeres in the absence of either telomerase activity or ALT (detected
of combination therapies is urgently needed. Agents targeting these key pathways and the evaluation only be beneficial when given in combination with lapse [97, 98]. Therefore, TMM targeting strategies will occurrence of RAS/TP53 pathway alterations with a further studies have shown that although TERT alterations and MYCN amplification do co-exist in a small proportion of cases, there is no overlap between the telomerase expressing and ALT positive neuroblastoma [47]. This is in keeping with a recent publication also showing that ATRX mutations and MYCN amplification are synthetically lethal in neuroblastoma [91]. Taken together, the evidence in neuroblastoma to date is that the presence of either ALT or telomerase activation is associated with differing distinct genetic drivers and occurs in mutually exclusive nature [4, 5].

Although data thus far indicates that subgroups of neuroblastoma are driven by either telomerase or ALT activation, it is highly likely that a selective pressure targeting one TMM will support the emergence of an alternative mechanism. In multiple other cancer subtypes it has been shown that long-term telomerase inhibition results in the emergence of features consistent with ALT [92–94]. Conversely, with the development of ALT targeted therapeutics it is probable that an up-regulation of telomerase will be seen. However, encouragingly, following ATRX LoF, it appears that ALT is a necessary adaptation for cancer cell survival, and that reactivation of telomerase activity cannot overcome endogenous telomere dysfunction [33].

Finally although it has been shown that activation of a TMM is the key determinant of poor outcome in neuroblastoma, the key drivers of TMM’s in neuroblastoma; MYCN amplification and genetic alterations in ATRX are also associated with distinct patterns of wider transcriptional activation which drive malignant transformation [91, 95, 96]. Furthermore it is known that the co-occurrence of RAS/TP53 pathway alterations with a TMM is associated with a particularly dismal outcome [6] and accordingly, alterations in the RAS and TP53 pathways are enriched at the time of neuroblastoma relapse [97, 98]. Therefore, TMM targeting strategies will only be beneficial when given in combination with agents targeting these key pathways and the evaluation of combination therapies is urgently needed.

Conclusion

Despite evidence that telomere maintenance is a key driver of aggressive biology in neuroblastoma, clinical translation of novel therapeutics specifically targeting telomere maintenance remains extremely challenging. The only compound to make it into paediatric clinical trials so far, imetelstat is excessively toxic and pre-clinical data on other compounds targeting telomerase activity is extremely limited. The most promising telomerase targeting candidate to date is 6-thio-dG, however this agent is yet to make it into clinical trials. It must also be noted that in an aggressive malignancy such as neuroblastoma, rapid development of resistance to single-agent targeting of telomerase activation is highly likely and that combination therapies will be needed to overcome this. Also, in the case of MYCN amplified tumours, transcriptional up-regulation of TERT is only one of many oncogenic programmes up-regulated in MYCN amplified neuroblastoma cells.

For ALT driven cancers, there is preliminary data supporting specific roles of a handful of targeted therapeutic approaches but a dearth of robust evidence of pre-clinical efficacy specifically for neuroblastoma. The combination of ATM inhibition with chemotherapy is currently the most promising option for ALT neuroblastoma, although regimens combining cytotoxic chemotherapy agents and inhibition of master upstream regulators of DNA damage repair such as ATM are likely to be prone to significant toxicities.

Despite these challenges, the development of effective new strategies for neuroblastoma by either selective targeting of telomerase or ALT offers great potential to treat the underlying drivers of aggressive disease biology, and is applicable to the greater proportion of neuroblastoma patients. Furthermore, the hypothesis that TMM targeting strategies may be particularly effective in neuroblastoma is supported by the fact that significantly fewer mutations are found in neuroblastoma in comparison to adult malignancies, which often arise due to an accumulation of oncogenic mutations over time [99].

In addition, as recent data identifies an ‘ultra-high risk’ group of neuroblastoma patients with both telomere maintenance and mutations in RAS and TP53 pathway genes [6], rational combinations of telomere targeting agents with other targeted therapeutics must be sought. As pre-clinical data develops, rationally designed paediatric clinical trials will be required to personalise therapy to simultaneously target multiple drivers of aggressive biology in an individual patient.

As our understanding of the molecular drivers of fatal neuroblastoma has significantly expanded in recent years, pre-clinical research efforts must now focus on translating this knowledge into effective, less toxic new therapies for children with neuroblastoma.

Abbreviations

TERT: Telomerase reverse transcriptase; ALT: Alternative lengthening of telomeres; TMM: Telomere maintenance mechanism; hITR: Human telomerase RNA; LoF: Loss of function; ATRX: Alpha Thalassemia mental Retardation-X linked; HR: Homologous recombination; MRN: Mre11-RAD50-Nbs1; PML: Pro-myelocytic Leukaemia; TERRA: RNA TElomeric Repeat-containing RNA; DAXX: Death domain associated protein; TP53: Tumour protein 53; RB1: Retinoblastoma 1; TRAP: Telomere repeat amplification protocol; FFPE: Formalin fixed paraffin embedded; ATM: Ataxia telangiectasia mutated; ATR: Ataxia Telangiectasia and Rad3 related; FISH: Fluorescence in-situ hybridisation

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Authors’ contributions
SLG and VP wrote the manuscript. SLG performed the in vitro experiments. In vivo experiments were performed by EP and YJ. LVM and PA critically appraised and edited the manuscript. LC supervised the study. The author(s) read and approved the final manuscript.

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Availability of data and materials
Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Ethics approval and consent to participate
This study did not involve human participants, data or tissue. For mouse experiments, protocols were approved and monitored by The Institute of Cancer Research Animal Welfare and Ethical Review Body (PPL 70/7945, later PPL P91525232), in compliance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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