

## **Chemical exposure and infant leukaemia: development of an adverse outcome pathway (AOP) for aetiology and risk assessment research<sup>1</sup>**

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## **Abstract**

Infant leukaemia (<1 year old) is a rare disease of an *in utero* origin at an early phase of foetal development. Rearrangements of the mixed-lineage leukemia (*MLL*) gene producing abnormal fusion proteins are the most frequent genetic/molecular findings in infant B cell-acute lymphoblastic leukaemia (ALL). In small epidemiological studies, mother/foetus exposures to some chemicals including pesticides have been associated with infant leukaemia; however, the strength of evidence and power of these studies are weak at best. Experimental *in vitro* or *in vivo* models do not sufficiently recapitulate the human disease and regulatory toxicology studies are unlikely to capture this kind of hazard. Here we develop an adverse outcome pathway (AOP) based substantially on an analogous disease – secondary acute leukaemia caused by the topoisomerase II (topo II) poison etoposide – and on cellular and animal models. The hallmark of the AOP is the formation of *MLL* gene rearrangements *via* topo II poisoning, leading to fusion genes and ultimately acute leukaemia by global (epi)genetic dysregulation. The AOP condenses molecular, pathological, regulatory and clinical knowledge in a pragmatic, transparent and weight of evidence-based framework. This facilitates the interpretation and integration of epidemiological studies in the process of risk assessment by defining the biologically plausible causative mechanism(s). The AOP identified important gaps in the knowledge relevant to aetiology and risk assessment, including the specific embryonic target cell during the short and spatially-restricted period of susceptibility, and the role of (epi)genetic features modifying the initiation and progression of the disease. Furthermore, the suggested AOP informs on a potential Integrated Approach to Testing and Assessment (IATA) to address the risk caused by environmental chemicals in the future.

**Keywords:** infant leukaemia; *MLL* fusion products; risk assessment; etoposide; DNA topoisomerase II

**Abbreviations:** ALL, acute lymphoblastic leukaemia; HSPC, hematopoietic stem and progenitor cell; IATA, Integrated Approach to Testing and Assessment; *MLL*, mixed-lineage or myeloid/lymphoid leukaemia (gene); *MLL*-r, rearrangements of *MLL* gene; t-AL, therapy-associated acute leukaemia; topo II, DNA topoisomerase II.

## INTRODUCTION

Do chemicals in general and pesticides in particular cause leukaemia in early childhood? That is the question raised by epidemiological investigations that, by their nature, are necessarily challenging and currently yield only “weak signals” of possible associations (Hernandez and Menendez, 2015). Although acute leukaemia is the most common cancer diagnosed in children aged less than 15 years, it is actually a phenotypically- and genetically-heterogeneous disease of immature hematopoietic stem and progenitor cells (HSPCs). Another important challenge to epidemiological studies is the complexity of delineating infant leukaemia from childhood leukaemia, as they represent a collection of aetiologically and mechanistically different diseases with variable natural histories and outcomes. In particular, infant leukaemia is an early-onset, rapidly developing and prognostically dismal form of paediatric leukaemia, which can also be considered as a developmental disease of immature HSPCs.

The low incidence of infant cancer represents a challenge for epidemiological correlative studies. The potential role of *in utero* exposure to environmental chemicals, including pesticides, in initiating paediatric leukaemia was originally suspected on the basis of epidemiological studies ((Ntzani et al., 2012; Hernandez and Menendez, 2015). The association between prenatal exposure (via occupational exposure of the parents) and paediatric leukaemia was observed both for ALL and acute myeloid leukaemia (AML), but recognising the considerable uncertainty in regard to the assessment of pesticide exposure. Besides an association with the generic term ‘pesticides’, the data did not allow to conclude about a single pesticide, or a specific pesticide group, associated with a significant risk of paediatric leukaemia. Also, almost all the available studies on the topic included both infant leukaemia and childhood leukaemia in the same diagnosis. Because only very few studies examined the risk of pesticide exposure with infant leukaemia as a separate entity,(Pombo-de-Oliveira et al., 2006; Ferreira et al., 2013), data thus far do not allow any firm conclusions to be drawn.

However, there is a form of human acute leukaemia caused by certain anticancer drugs that can be divided into two well-defined groups according to whether the patient has received alkylating agents or drugs binding to the enzyme DNA-topoisomerase, such as epipodophyllotoxins and anthracyclines (Mistry et al., 2005; Felix et al., 2006; Leone et al., 2007). This is the so-called treatment-associated acute leukaemia, whereby anticancer agents have been considered to play an aetiological role in promoting the driver genetic oncogenic events (Cowell and Austin, 2012; Ezoe, 2012; Pendleton et al., 2014; Gole and Wiesmuller, 2015). The most well-known

chemotherapeutic agent is etoposide (an epipodophyllotoxin), which can induce the formation of a fusion gene as a result of a chromosomal translocation involving the mixed-lineage leukaemia (*MLL*, *KMT2A*) gene and other partner genes through topoisomerase II (topo II) “poisoning”.

The Adverse Outcome Pathway (AOP) is a conceptual framework linking the Molecular Initiating Event (MIE) to a final Adverse Outcome (AO). It provides a basis for multiple-level data integration by defining the intermediate essential Key Events (KEs) that link the MIE to the AO, and providing a scientifically firm rationale for the KE relationship (KER) (OECD, 2013). In this review, a panel of experts working on a European Food and Safety Authority (EFSA) mandate surveyed the scientific background, assisted by a systematic literature review (Choi et al 2016), to build a biologically plausible and experimentally supported AOP linking *in utero* poisoning of topo II enzyme with the ultimate AO “infant leukaemia” (see also <https://aopwiki.org/aops/202>). Finally, we outline potential uses of this AOP for identifying future research needs and for regulatory decisions regarding chemicals with appropriate experimental findings.

### **What is infant leukaemia?**

B cell-ALL is the most frequent cancer in children. Infant leukaemia is a rare haematological disease with an incidence of 1 in 10<sup>6</sup> newborns, accounting for 10% of all B cell-ALLs in children younger than 15 years, manifesting soon after birth (<1 year) and displaying an intermediate prognosis except for some cytogenetic subgroups such as *MLL*-rearranged (*MLL*-r) B cell-ALL, which remains an outlier high-risk group having a poor prognosis (Sanjuan-Pla et al., 2015). Compared with the more frequent childhood leukaemias, infant leukaemia shows distinct features (see Table 1 for a more comprehensive comparison):

- An early neonatal manifestation suggests an *in utero* initiation as an ‘intrauterine developmental disease’ (Greaves, 2015; Sanjuan-Pla et al., 2015);
- Rearrangements of the *MLL* gene on the q23 band of chromosome 11 as the hallmark genetic abnormality (Joannides and Grimwade, 2010).
- However, *MLL* is not the only translocation gene<sup>2</sup>. Whereas about 60–80% of infant ALL carry an *MLL*-r (Sam et al., 2012; Jansen et al., 2007), for infant acute myeloid leukaemia (AML) the percentage of *MLL*-r is lower than 40%;

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<sup>2</sup> Even if *MLL* is not present in 100% of infant leukaemias, the ‘*MLL*-rearranged (*MLL*-r) infant leukaemia’, especially *MLL*-r B-ALL, is taken here as a model for the disease principally because of the quantity of scientific evidence.

- The *MLL-r* occurs at an early stage of development, with the target cells (still unidentified) being likely the HSPCs in foetal liver and/or pre-haematopoietic mesodermal foetal precursors (Bueno et al., 2009; Menendez et al., 2009);
- Infant *MLL-r* leukaemia has the least number of somatic mutations among all the sequenced cancers (1.3 vs 6.5/case; Andersson et al., 2015; Dobbins et al., 2013), pointing to the lack of a “second hit” assumed in the classic carcinogenesis paradigm.

The overall scientific evidence, including the stable genome of patients, suggests that infant leukaemia originates from one “big-hit” occurring during a critical developmental window of HSPC vulnerability (Andersson et al., 2013; Greaves, 2015). In contrast to the “two-hit” model of the adult and childhood leukaemias, infant leukaemia is a developmental disorder where the differentiation arrest and clonal expansion are a direct consequence of *in utero MLL* translocation in target HSPCs.

### **Secondary treatment-related acute leukaemia: a model for infant leukaemia?**

Secondary acute leukaemia carrying *MLL-r* is an adverse effect observed in patients treated with etoposide and a few other anticancer agents. Characteristics of the disease are in many ways analogous to those in infant leukaemia (Joannides et al., 2010, 2011) (Table 1). This so-called therapy-associated acute leukaemia (t-AL) in adults is characterised by its short latency, <2 years between the treatment of the primary malignancy with epipodophyllotoxins and the clinical diagnosis of the secondary disease, and by the poor prognosis (Cowell and Austin, 2012; Ezoë, 2012; Pendleton et al., 2014). It is recognised that the *MLL-r* fusion genes are caused by etoposide, other epipodophyllotoxins or anthracyclines, because *MLL-r* have not been detected in bone marrow samples banked before the initiation of the treatment for the first malignancy (Cowell and Austin, 2012; Pendleton et al., 2014). Overall, the evidence supporting the causal relationship between etoposide-induced topo II inhibition and further formation of cleavage complexes leading to *MLL-r* is strong and could be regarded as ‘beyond reasonable doubt’. Also, the breakpoints in *MLL* or partner genes fall within a few base pairs of a drug-induced enzyme-mediated DNA cleavage site (Cowell and Austin, 2012, Pendleton et al., 2014; Gole and Wiesmuller, 2015). All the above disease characteristics, *MLL-r*, short latency and poor prognosis, strongly suggest that infant leukaemia and treatment-related leukaemia are sufficiently similar to allow for inferences to be made regarding tentative aetiological factors, molecular events and disease progression and manifestation. Thus, etoposide can be considered as a model chemical for DNA topo II inhibition and *MLL-r* and it was used here as a tool compound to empirically support the linkage between MIE and AO in the AOP.

### *Etoposide*

There is abundant evidence on the interaction of etoposide with topo II enzymes, resulting in further chromosomal translocations (in particular *MLL-r*) at the cell culture level and in relation to treatment-related leukaemia (Cowell and Austin, 2012; Ezoe, 2012; Pendleton and Osheroff, 2014; Gole and Wiesmuller, 2015). Etoposide can induce *MLL-r* in different cell types. Interestingly, embryonic stem cells and their hematopoietic derivatives are much more sensitive than cord blood-derived CD34<sup>+</sup> cells to etoposide induced *MLL-r*. In addition, undifferentiated human embryonic stem cells (hESCs) were concurrently predisposed to acute cell death (Bueno et al., 2009). The effects of etoposide in various model systems will be described in detail in the description of the developed AOP.

There should be some information about the concentration-response relationships of the selected tool chemical in the development of the AOP. Molecular dose-response modelling of etoposide-induced DNA damage response, based on comprehensive *in vitro* high content imaging in the HT1080 cell model, was developed by Li et al. (2014). The model was based on the hypothesis that cells are capable of clearing low-level DNA damage with existing repair capacity; however, when the number of double strand breaks (DSBs) exceeds a threshold value, ataxia telangiectasia mutated (ATM) is recruited and becomes fully activated through a reversible mechanism, leading to elevated repair capacity as a result of phosphorylation (activation) of several target proteins, including p53 and other tumour suppressor proteins. The model was able to quantitatively capture the dose-response relationships of a number of markers observed with etoposide. Especially interesting are the dose-response relationships for activation of p53 and the formation of micronuclei in the target cell model, which indicate point-of-departure concentrations of etoposide in the range of 0.01 to 0.1  $\mu\text{M}$  (Li et al., 2014). This range is in agreement with the finding that in human foetal liver CD34<sup>+</sup> cells an increase in DSBs was observed at a concentration of 0.14  $\mu\text{M}$  (Bueno et al., 2009) and *MLL-r* were detectable by FISH or flow cytometry at higher concentrations (Moneypenny et al., 2006).

### **The general structure of the AOP**

The AOP concept has been developed to incorporate the available information into a wider context, and to integrate various pieces of scientific information into a structurally coherent and biologically plausible framework of identifiable, not necessarily fully developed, essential key events that comprise linear and sequential processes in the way from the MIE through a variable number of

intermediary steps, that is, key events, to the AO. Information to build the AOP may come from any type of scientific study and span various levels of biological organization, from molecular to organism levels. This chain of biological events is considered relevant for risk assessment. The AOP is not the final goal in itself; instead, it could serve several purposes, for example, identification of crucial targets and processes and their employment in the development of novel tests and screening systems, and, ultimately, the elaboration of prioritisation strategies and research programs (OECD, 2012; Vinken, 2013). Although the AOP as a concept is similar to the mode of action, since both take into account mechanistic information, one major difference is that a mode of action focuses on the details specific to a particular chemical (i.e., toxicokinetic and toxicodynamic information), whereas the AOPs represent the chemical-agnostic portions of toxicity pathways (i.e., toxicodynamic information) to increase the generalisability of their application from early key events to overt toxicity (Edwards et al., 2016).

### **The AOP for Infant Leukaemia**

The final structure of the developed AOP is presented in Fig 1 and elaborated in detail in the subsequent sections. The first key event, the MIE, is the *in utero* DNA topo II inhibition, ‘poisoning’, and further stabilisation of the normally transient reaction DNA-topo II intermediate in which the enzyme is covalently bound to DNA. If topo II poisons target the *MLL* breakpoint cluster region, the *MLL* gene can be cleaved *via* DNA DSBs. If the DNA lesions are not correctly repaired by the error-prone non-homologous end joining (NHEJ) repair system, DSBs accumulate. Thus, the *MLL* gene can be rearranged with a number of other genes that have been simultaneously cleaved, resulting in the formation of fusion genes, which represent the second key event. Molecular and cellular processes of the first key event relationship (KER1) have been clarified at least on a general level. The product of the fusion gene encompasses many preserved and acquired functions of the fusion partners associated with differentiation block of HSPCs and expansion of clones expressing the fusion product (KER2). Originally an additional (third) key event, differentiation block and clonal expansion, was envisaged, but ultimately (at least for the time being), we decided that the information is in effect contained in the fusion protein and, finally, the process (KER2) from the rearranged fusion gene leads to the manifest leukaemia (the third key event or adverse outcome, AO). The precise molecular and cellular processes behind KER2 remain incompletely understood, but changes in gene activation and repression as well as in epigenetic regulation in a hypothetical “permissible” cellular environment, restricted in time and space, likely play a decisive role (Greaves, 2015; Sanjuan-Pla et al., 2015).

In summary, current scientific evidence, including the stable genome of the patients, suggests that infant leukaemia originates from one “big-hit” occurring during a critical developmental window of stem cell vulnerability (Andersson et al., 2013; Greaves, 2015). Therefore, the totality of evidence suggests the essential role of the formation of *MLL-AF4* (or other partner) fusion gene (and product) in causing pleiotropic effects in the affected cell, which is then directed to the obligatory pathway to the adverse outcome of infant leukaemia (see KER2).

The AOP is presented in detail in the following sections. As becomes evident, the proposed AOP is supported by convincing inferential evidence using etoposide as a tool compound to empirically support the linkage between the proposed MIE and the AO.

### **Molecular Initiating Event (MIE): *In utero* exposure to DNA topoisomerase II “poisons”**

DNA topoisomerases are ubiquitous enzymes that control the integrity of double-stranded DNA. They are thus key enzymes at all levels of living organisms. While type II topoisomerases are essential for the survival of proliferating cells, they can also contribute significantly to genotoxic effects *via* accumulation of DNA DSBs that, if not leading to cell death, may result in chromosomal translocations in the surviving cell population (McClendon et al., 2007).

Topo II enzymes have several crucial functions in DNA replication, transcription, repair and chromatin remodelling, maintaining DNA integrity and topology. Because the enzyme functions by passing an intact double helix through a transient double-stranded break, any disturbance in its function, for example by chemical inhibitors, could have a profound effect on genomic stability, resulting in activation of DNA damage response, initiation of apoptosis and ultimate cell death, or on gene and chromosomal damage and its repair. A DSB and error-prone NHEJ DNA repair mechanism may lead to gene rearrangements, chromosomal translocations and consequently gene fusion (see Fig 1). Characteristics of topo II enzymes and their functions and inhibitions have been extensively reviewed (Cowell and Austin, 2012; Pendleton et al., 2014; Ketron and Osheroff, 2014).

Compounds that increase the rate of DNA cleavage and decrease the rate of DNA religation by topo II enzyme are often referred to as topo II ‘poisons’ (Nitiss, 2009). Such “poisons” disturb the normal enzyme function and cause a ‘hanging DSB’ at a specified DNA sequence, thus creating the potential for fusion gene formation. The above description of the MIE is significant because there are 3 different types of topo II inhibitors, including competitive inhibitors that prevent the function of the enzyme and cause cell death, and interfacial and covalent inhibitors that may cause–



depending on the situation—other effects on DNA damage response such as chromosomal rearrangements (Pendleton et al., 2014; Lu et al., 2015). A further prerequisite for the specific outcome, that is, creation of chromosomal rearrangement, is that topo II poisoning occurs in an especially vulnerable and correct “hot spot” in the *MLL* locus, in the specific target cell vulnerable to transformation (Gole and Wiesmuller, 2015).

#### *How the MIE is measured*

Measurement of the inhibition of topo II enzymes is complicated by the existence of different molecular mechanisms (see above). However, some assays are used to screen topo II “poisons”, including cell-free decatenation assay (Schroeter et al., 2015). The most important mode, the cleavage activity of topo II, can be studied *in vitro* using a human recombinant enzyme and an appropriate double-stranded plasmid as a target to quantitate DSBs (Fortune and Osheroff, 1998). Cleavage can also be indirectly detected by measuring the outcomes it elicits with regards to indicators of DNA damage response, such as ATM activity, p53 expression,  $\gamma$ H2AX (phosphorylated form of the histone H2AX) or the Comet assay (Li et al., 2014, Schroeter et al., 2015, Castano et al., 2016).

### **How DNA topoisomerase II inhibition leads to *in utero* *MLL* chromosomal translocation: Key Event Relationship 1 (KER1)**

#### *Formation of the fusion gene*

Certain topo II poisons stabilise (and trap) the intermediate “cleavage complex”, preventing the religation with appropriate DNA strands. Covalent DNA end-bound topo II protein is digested and a hanging end is created. The same process occurs in the translocation partner gene. The hanging ends of both genes are processed and two heterologous ends are subsequently joined by NHEJ (Cowell and Austin, 2012). There is evidence that this inappropriate joining of ‘hanging ends’ occurs in the same transcriptional factory (hub), and the result is a fusion gene and ultimately a chimeric protein product (Cowell & Austin, 2012; Pendleton et al., 2014; Sanjuan-Pla et al., 2015). The first part of this description has not been shown in the putative target cell, which is still not unequivocally identified; but for the second part, there is ample evidence of formation of *MLL-AF4* fusion product as a result of a very early chromosomal translocation and re-joining. It is notable that the simultaneous induction of specific DSBs in introns of the *MLL* gene and two different translocation partners (AF4 and AF9) by engineered nucleases in human HSPCs resulted in specific ‘patient-like’ chromosomal translocations (Breese et al., 2016).

### *Embryo-foetal origin of a fusion gene*

The occurrence of *MLL-r* at a very early foetal development stage is highly probable on the basis of neonatal blood spot analysis and by the near 100% concordance rate of infant leukaemia in monozygotic twins (Ford et al., 1993; Gale et al., 1997).

Experimentally, the induction of DNA breaks and *MLL-r* by etoposide and some bioflavonoids has been demonstrated in human foetal liver HSPCs, hESCs and in cord blood mononuclear cells (Ishii et al., 2002; Blanco et al., 2004; Money Penny et al., 2006; Bueno et al., 2009; Menendez et al., 2009), which clearly shows that topo II-associated *MLL-r* rearrangements are produced in prenatal and neonatal human cells.

Etoposide treatment in mice at day 13.5 of pregnancy induces *mll* breakage in foetal liver HSPCs *in utero*, but *MLL-r* fusion mRNAs were detected only in mice that were defective in the DNA damage response (ATM-knockout mice). A fusion gene analogous to *MLL-AF4* was not detectable in wild-type mice. A statistically significant increase (about 6-fold) in DSBs in the *mll* gene of isolated foetal liver HSPCs was observed after a single dose of 1 mg/kg to pregnant mice. A clear activation of DNA damage response was observed at the dose of 10 mg/kg (Nanya et al., 2016).

### ***In utero MLL chromosomal translocation: Key Event 1 (KE1)***

#### *Fusion genes and their products*

Rearrangements of the *MLL* gene, located on chromosome 11 (11q23), are the genetic hallmark of most infant leukaemias (Meyer et al., 2013; Sanjuan-Pla et al., 2015). The *MLL* gene spans a chromosomal fragile site (that is, a region of the genome susceptible to breakage under conditions of replication stress), and chemical interference with topo II activity may promote fragile site instability. More than 95% of the *MLL-r* occur within a ~7.3 kb breakpoint cluster region (bcr) that is distributed in two clusters, with breaks from t-AL and infant leukaemia being located at the telomeric part of the *MLL* bcr (Gole and Wiesmuller, 2015). *MLL* breakpoint site sequences in the t-AL fall within a few base pairs of an etoposide-induced enzyme-mediated DNA cleavage site (Cowell and Austin, 2012; Meyer et al., 2013). Although rearrangements associated with infant leukaemias are often more complex than those observed in t-AL, many are nevertheless associated with stable topo II-mediated DNA cleaved sites. While many of these findings are indirect regarding infant leukaemia, they are nonetheless rather persuasive in this respect.

DNA breakage within the *MLL* gene can lead to rearrangement with over 90 partner genes, leading to many translocations and fusion partners (Meyer et al., 2013). In principle, all *MLL* fusion genes are potential initiating drivers, although clinical studies have shown preponderance with infant leukaemia for only a few of these rearrangements. For infants diagnosed with ALL, approximately 60–80% carry an *MLL* rearrangement (Sam et al., 2012; Jansen et al., 2007), with principal fusion partners being *AF4* (41%), *ENL* (18%), *AF9* (11%) or another partner gene (10%). In particular, the fusion gene *MLL-AF4* shows a specific and consistent relationship with the disease (Menendez et al., 2009); however, it has been difficult to reproduce an overt disease resulting from this rearrangement in animal models (see later sections).

Menendez et al. (2009) showed that *MLL-AF4* fusion gene is present and expressed in bone marrow mesenchymal stem cells in infant patients with t(4;11) B cell-ALL. However, other paediatric B cell-ALL-specific translocations/gene fusions were never found in this cell population. This suggests that the origin of the fusion gene in infant B cell-ALL is likely prehaematopoietic. Consequently, the target cell for transformation may be an early prehaematopoietic mesodermal precursor, a haematopoietic stem cell or a haematopoietic progenitor cell residing mainly in the liver (Greaves, 2015; Sanjuan-Pla et al., 2015).

#### *Fusion proteins and their functions*

The *MLL* gene encodes a lysine-specific histone methyltransferase 2A, a protein homologous to the *Drosophila trithorax* gene, which has important functions in embryogenesis and haematopoiesis (Ernest et al., 2004, Hess et al., 1997). *MLL* protein (complexed with a large number of other protein factors) serves as an epigenetic transcriptional activator or repressor *via* its binding to promoter regions of active genes, marking these regions by covalent histone modifications (Sanjuan-Pla et al., 2015). Chromosomal translocations affecting the *MLL* gene result in fusion genes that destroy the intrinsic control mechanisms of the *MLL* protein. The resulting aberrant functions involve promoter hyperactivation and reacquisition of stem cell features (Marschalek, 2010; Sanjuan-Pla et al., 2015).

#### *How the KEI is measured*

The presence and structure of a fusion gene can be identified using PCR or related techniques. Mapping of cleavage sites in the gene requires genomic DNA. In cells or tissues, the detection of a fusion gene is possible by suitable immunofluorescence techniques. Assays measuring chromosomal aberrations, micronuclei or DNA chromosome damage (e.g., Comet assay) may

indirectly identify the KE through its consequences in experimental systems *in vitro* and *in vivo*; the degree of accuracy of such identification cannot be evaluated presently. Comprehensive genomic tool platforms have been developed to screen genomic changes in leukaemic diseases (McKerrell et al., 2016).

### **How *in utero* MLL-r leads to infant leukaemia: Key Event Relationship 2 (KER2)**

Propagation of a leukaemic cell clone requires both blockage of differentiation to more mature cells and ability to expand in an uncontrolled manner. It is probable that the potential of both differentiation blockage and clonal expansion are inherent properties of the *MLL-r* fusion product, based on the preservation of some original functions (even if in a modified form) and on the gain of other functions as a result of sequences from the new fusion partner gene (Marschalek, 2010; Sanjuan-Pla et al., 2015). As the formation of the *MLL-r* fusion genes and their protein products are intimately involved both in the blocked differentiation of HSPCs and the expansion of the fusion gene-carrying clone, it is believed that the fusion gene product blocks cell differentiation by inhibiting the normal transcriptional programs and recruiting repressor molecules such as histone modifying enzymes (Greaves, 2002; Teitell and Pandolfi, 2009). Furthermore, the fusion gene product activates other key target genes, which ultimately leads to the propagation of transformed cell lines without normal restrictions (Greaves, 2015; Sanjuan-Pla et al., 2015).

#### *Molecular mechanisms*

The *MLL* gene is the most common translocation gene identified in infant leukaemia. The N-terminal part of *MLL* becomes fused in-frame to one of a large number of fusion partners, but in most cases, this fusion occurs between the N-terminal *MLL* and *AF4*, *AF6*, *AF9*, *AF10*, or *ENL* (Krivtsov and Armstrong, 2007; Meyer et al., 2013). The N-terminal *MLL* remaining in the fusion gene contains a motif for DNA-binding with target genes controlled by *MLL* irrespective of the normal location of the C-terminal partner (Farooq et al., 2016).

The role of the fusion partners in the process of leukaemogenesis has not been completely elucidated. Table 2 lists some of the salient functional features of the most frequent fusion partners of the *MLL* gene. Normally, all of them participate in chromatin modifying complexes, for example, super elongation complex, which act on the transcriptional regulation of target genes such as homeobox A (*HOXA*) cluster genes or myeloid ectopic viral integration site 1 homolog (*MEIS1*). This highly regulated process is disturbed by the presence of *MLL* fusion proteins and it seems

probable that different fusion partners are distinct entities with variable modulatory effect on signalling pathways in leukaemic cells (Marschalek, 2010).

Many fusion proteins have been shown to recruit disruptor of telomeric silencing 1-like (DOT1L or officially KMT4, a histone methyltransferase that methylates lysine 79 located within the globular domain of histone H3, H3K79) to the promoters of *MLL* target genes. This recruitment seems to be a common feature of many oncogenic *MLL* fusion proteins and results in abnormal H3K79 methylation and overexpression of several *MLL* target genes, such as *HOXA* genes coding for transcription factors involved in body patterning and haematopoiesis (Chen and Armstrong, 2015). Although DOT1L is not genetically altered in the disease *per se*, its mislocated enzymatic activity is a direct consequence of the chromosomal translocation. Thus, DOT1L has been proposed to be a catalytic driver of leukaemogenesis (Chen and Armstrong, 2015; Kerry et al., 2017). The enzymatic activity of DOT1L is critical to the occurrence of *MLL* because methyltransferase-deficient DOT1L is capable of suppressing growth of *MLL*-rearranged cells. A small-molecule inhibitor of DOT1L inhibits cellular H3K79 methylation, blocks leukaemogenic gene expression, and selectively kills cultured cells bearing *MLL* translocations (Chen and Armstrong, 2015). One of the target genes of DOT1L is *BCL-2*, belonging to a family of anti-apoptotic genes, which maintains the survival of the *MLL*-rearranged cells (Benito et al., 2015; Godfrey et al., 2017). Expression of *BCL-2* is high in human *MLL-AF4* leukaemia cells from a large number of patients and *BCL2* inhibition with specific antagonists may make *MLL*-r infant leukaemia sensitive to current treatment. Fig 2 provides a schematic representation of potential molecular pathways.

#### *Potential significance of secondary oncogenic events*

Recurrent activating mutations in the components of the phosphoinositide 3-kinase (PI3K)-RAS signalling pathway have been detected in almost 50% of the tested *MLL*-r ALLs (Andersson et al., 2015). RAS mutations, possibly of prenatal origin, have been demonstrated also in other studies of infant leukaemia, with frequencies of 15–25 % in cases (Driessen et al., 2013; Prelle et al., 2013; Emerenciano et al., 2015). While the somatic dysregulation of RAS is a primary driver of cancer, germline mutations in genes that encode components of the RAS signalling pathway are involved in a number of developmental disorders (Niemeyer, 2014). However, RAS mutations do not seem to be driver mutations in infant leukaemia, but instead may aid disease onset by accelerating the initial expansion of cells (Emerenciano et al., 2015).

Overall, the activation of the RAS pathway could support the extremely rapid progression of infant leukaemia. Along this line, RAS activation may represent a factor modulating (i.e., increasing) the progression and severity of the adverse outcome, rather than a necessary key event (second hit) for infant leukaemia. In the transgenic *MLL-AF4* mouse model, activated K-RAS accelerated disease onset with a short latency (Tamai et al., 2011), possibly by augmenting the upregulation of *HoxA9*. In a recent study by Prieto et al. (2016), the activated K-RAS enhanced extramedullary haematopoiesis of *MLL-AF4*-expressing cell lines and cord blood-derived CD34<sup>+</sup> HSPCs, which was associated with leukocytosis and central nervous system infiltration. Both are hallmarks of infant *MLL-AF4* leukaemia. However, K-RAS activation was insufficient to initiate leukaemia, supporting the involvement of the RAS pathway as an important modifying factor in infant leukaemia. It has also been demonstrated that *MLL-AF6* fusion product sequesters AF6 into the nucleus to trigger RAS activation in myeloid leukaemia cells, which indicates that RAS may play a crucial oncogenic role in AML (Manara et al., 2014). The possibility that *MLL* fusions render cells susceptible to additional chromosomal damage upon exposure to etoposide was examined by introducing *MLL-AF4* and *AF4-MLL* via CRISPR/Cas9-genome editing in HEK293 cells, as a model to study *MLL* fusion-mediated DNA-DSB formation/repair (Castano et al., 2016). The authors concluded that the expression of fusion genes does not influence DNA damage signalling or DNA-DSB repair.

#### *Experimental models to support the significance of rearrangements*

There are several animal models in which *MLL-AF4* fusion gene has been expressed (Chen et al., 2006; Metzler et al., 2006; Krivtsov et al., 2008; Bursen et al., 2008; Tamai et al., 2011). Leukaemia is ultimately developed in all the models, but latency is very protracted (see Sanjuan-Pla et al., 2015). Regardless, one could conclude that the expression of the *MLL-AF4* (or its reciprocal) fusion gene in these models is capable of triggering leukaemia, but it is unknown whether facilitating or additional changes are required during the long latency in the mouse.

Lin et al. (2016) designed a fusion gene between human *MLL* and murine *af4* and demonstrated that it could transform—via retroviral transduction—human CD34<sup>+</sup> cells to generate pro-B-ALL with all the characteristic features of the *MLL-AF4* infant leukaemia. This finding suggests that the *MLL-af4* protein retains similar molecular properties to the native human *MLL-AF4*. The authors claim that the ability of *MLL-af4* to transform is due to the high-titre viral production. However, previous reports using *MLL-AF4* also demonstrated proper transgene expression in CD34<sup>+</sup> HSPCs.

Therefore, this new model, despite its hybrid nature, offers the first real platform to experimentally study infant leukaemia.

Transcription activator-like effector nuclease (TALEN)-mediated genome editing was used to generate endogenous *MLL-AF9* and *MLL-ENL* oncogenes in primary human HSPCs derived from human umbilical cord plasma (Buechele et al., 2015). Engineered HSPCs displayed altered *in vitro* growth potential and induced acute leukaemias following transplantation in immunocompromised mice at a mean latency of 16 weeks. These leukaemias displayed phenotypic and morphologic similarities with patient leukaemia blasts, expressed elevated levels of crucial *MLL*-fusion partner target genes, displayed heightened sensitivity to DOT1L inhibition, and demonstrated increased oncogenic potential *ex vivo* and in secondary transplant assays.

#### **Adverse Outcome (AO): Infant leukaemia**

Symptoms of leukaemia–thrombocytopaenia resulting in enhanced sensitivity to bruising and bleeding, anaemia with pallor and fatigue, neutropaenia associated with increased susceptibility to infections—are principally due to the displacement of normal haematopoiesis by expansion of leukaemia cells. Leukaemic infiltration of the brain is common at diagnosis of the infant leukaemia (Hunger and Mulligham, 2015).

Clinical diagnosis is based on clinical symptoms and identification of leukaemia cells and altered blood cell counts by routine haematological methods. Following clinical diagnosis, methods for refined diagnosis include bone marrow aspirates for immunophenotypic analyses and cytogenetic assays for molecular stratification. Differentiation between B- and T-ALL and AML is of significance because particularly pro-B-ALL with the *MLL-AF4* rearrangement is the major form of infant leukaemia and its characteristics are of importance in judging the relevance of various experimental models for the human disease (see Lin et al., 2016).

The disease is not known in animals, and artificial animal models able to reproduce the disease have limitations. Bardini et al. (2015) have however developed a xenograph mouse model with transplanted patient *MLL-AF4*-involving leukoblasts. Other models developed by Breese et al. (2015) and Lin et al. (2016) are promising, but not yet extensively studied regarding their relevance.

Under the OECD conceptual framework for the regulatory hazard assessment of chemicals with respect to mammalian toxicity, the carcinogenicity assays (OECD TG 451 and TG 453) and the

extended one generation test (OECD TG 443) include endpoints that can potentially explore leukaemia as the AO; however, considerations should be made on the specificity of the disease to humans.

### **Evidence for Chemical Initiation of the AOP**

The AOP conceptual framework is intended to cover only the toxicodynamic part of the toxicity pathway and is therefore chemically agnostic. Tool chemicals are however necessary to provide the empirical support for the KERs and, for this reason, identification of chemicals able to interact with the MIE and to trigger the pathway up to the AO is a relevant part of the exercise. Indeed, the greater the evidence of different chemicals activating and triggering the pathway, the larger the strength of the AOP. A fairly large number of drugs, environmental chemicals and natural substances are identified as topo II poisons (Table 2). The anticancer drug etoposide has been used here as a tool chemical for building the AOP, mainly because of the availability and consistency of the empirical evidence. An especially interesting group is the bioflavonoids because they are suspected to cause infant leukaemia on the basis of epidemiological findings (see Hernandez and Menendez, 2015). Bioflavonoids are natural polyphenolic compounds in a large variety of plant-derived food items and some of them are used as herbal medicines. Topo II-mediated DNA cleavage has been linked to genistein, kaempferol, luteolin, myricetin and apigenin (Strick et al., 2000; Bandele and Osheroff, 2007; Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007; Azarova et al., 2010; Lopez-Lazaro et al., 2010), although the concentrations used in *in vitro* studies have been quite high. Several bioflavonoids are capable of inducing the cleavage of the *MLL* gene and producing a fusion gene (and its protein product) in human cell lines (Strick et al., 2000; Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007). The *in vitro* effects of bioflavonoids suggest a possible link between the intake of the bioflavonoid-rich diet in pregnant women and infant leukaemia (e.g., Azarova et al., 2010; Lanoue et al., 2010); however, epidemiological evidence in support of or refuting such a hypothesis is based on small studies (Ross et al., 1996; Spector et al., 2005).

Chlorpyrifos, a widely used organophosphate insecticide, and its metabolite chlorpyrifos oxon, have been shown to inhibit topo II activity *in vitro*, stabilise the topo II-DNA cleavage complex, cause DNA DSBs (as measured by the neutral Comet assay) and induce *MLL* gene rearrangements in human foetal liver-derived CD34<sup>+</sup> HSPCs, as detected by FISH (Lu et al., 2015). Accordingly, chlorpyrifos seems the most promising pesticide candidate for further in-depth studies. Previously, Borkhardt et al. (2003) published a case study of a single preterm female newborn with congenital



leukaemia and *MLL-r* whose mother had abused aerosolised permethrin during pregnancy because of arachnophobia. Further, incubation of permethrin in the BV173 cell line led to induction of *MLL* cleavage. Further epidemiological studies have found significant associations between infant leukaemia and maternal exposure to pyrethroids during pregnancy (Ferreira et al., 2013).

#### *Modifying conditions during the embryo-foetal period*

The available evidence suggests important differences in sensitivity to topo II inhibition among different cell types, depending on their inherent proliferative burden, the amount of the topo II enzymes and on physiological repair processes (see Sanjuan-Pla et al., 2015). Mesodermal precursors and HSPCs are rapidly dividing cells with a high content of topo II and, for these reasons, they can be a sensitive target during a critical developmental window (Hernandez and Menendez, 2016). In addition, human and animal studies have found higher levels of DNA damage and mutations induced by mutagens in foetuses/newborns than in adults (Udroiu et al., 2016). For instance, micronuclei assay studies conducted in untreated and chemical-treated foetuses and newborns show that both the baseline and chemically-induced micronuclei frequencies are higher in foetuses, pups and juvenile animals than in adults (Udroiu et al., 2016).

Also, murine studies point to the life stage-related susceptibility with regard to topo II poisoning. Nanya et al. (2015) demonstrated that mouse foetal liver HSPCs were more susceptible to etoposide than maternal bone marrow mononuclear cells.

#### **The potential significance of the AOP for future research**

The major incentive to develop this AOP was epidemiological hints, that is, significant albeit variable associations between paediatric leukaemia and pesticide exposure (Ntzani et al 2013; EFSA PPR Panel 2017). Contemplating the possibilities to strengthen the epidemiology findings, it became obvious that a vast gap exists between wide exposure to potential topo II poisons and the rarity of infant leukaemia. On the basis of studies in human adult and paediatric leukaemia, there is a large number of genetic, epigenetic and host factors potentially modifying the link between various chemical exposures and leukaemia. Because of the rarity of the disease, it is difficult to envisage, even partially, aetiological factors as of importance for the infant leukaemia.

In such a problematic situation, the AOP framework will serve in guiding scientific research, regulatory needs and testing strategy. This includes the exploration of topo II poison characteristics

of a chemical and, if the genotoxicity standard regulatory testing battery is negative, considerations should be made on the sensitivity of the cell system used in the assay (i.e., foetal liver HSPCs).

#### *Cellular and chromosomal targets and ensuing different fusion genes*

A prerequisite for the specific outcome, that is, creation of chromosomal rearrangements, is that topo II inhibition and poisoning has to occur in an especially vulnerable and correct hot spot in the *MLL* locus of target cells in order to acquire leukaemogenic potential. However, details of this process and how it happens are far from being fully elucidated (see Gole and Wiesmuller, 2015).

A target cell, that is, a leukaemia-initiating cell, has not been identified with sufficient confidence and consequently there is no target cell model to recapitulate the linkage between topo II poisoning and the production of DSBs in an appropriate target. Recently, Breese et al. (2015) produced specific chromosomal translocations in K562 cells and in primary HSPCs by the expression of engineered nucleases (TALENs) to induce simultaneous DSBs and patient-specific translocation breakpoints in the *MLL* gene and two different known translocation partners (AF4 and AF9). Lin et al. (2016) developed a model in which a rearrangement between human *MLL* and murine *af4* was retrovirally transduced into HSPCs, which developed into leukaemic cells with similar characteristics as infant leukaemia. Comparative studies between various cell models should shed light on important cellular elements and environments conducive to leukaemogenesis.

Relationships between different fusion genes and subsequent leukaemia types are incompletely understood. Although roughly 70–80 % of infant B-ALL carry *MLL-r*, the remaining 20–30 % of the cases lack *MLL-r*. In AML and T-ALL cases *MLL-r* are even rarer. Thus, it is quite possible that the AOP presented herein is strictly applicable only for the *MLL-AF4* B cell-ALL because a fusion partner may well confer important differences in the execution of the leukaemogenic pathway (Marschalek, 2010). However, we believe that such “variations on a theme” will be within the general framework of this AOP and thus it should be a useful template for other closely related pathways.

#### *Animal models*

The *MLL-AF4* knock-in mouse developed leukaemia only after a prolonged latency (Chen et al., 2006), thus not recapitulating the ‘pathognomonic’ feature of infant leukaemia. Other animal models have been developed with similar results (see Sanjuan-Pla et al., 2015). Thus, an appropriate experimental animal model for infant leukaemia is still needed.

The role of a reciprocal fusion gene *AF4-MLL* in leukaemias is controversial. While it has leukaemogenic potential in animal models (Bursen et al., 2010), it is expressed only in a portion of *MLL-AF4* patients (Andersson et al., 2015). The potential role of other reciprocal fusion genes remains to be elucidated.

*In utero* etoposide treatment of mice failed to induce leukaemogenesis (Nanya et al., 2015). Consequently, the envisaged linkage has not been empirically supported or rejected. However, it should be borne in mind that while etoposide does indeed induce a large number of *MLL-r*, most of them occur within non-coding regions and therefore do not elicit any direct oncogenic consequences. An *MLL-AF4* in-frame fusion is a rare event that needs to occur in a target cell within a relatively small and spatially-restricted cell population during the appropriate, epigenetically plastic, developmental window. These requirements make it difficult to empirically support this process.

#### *Better epidemiological studies are needed*

There is limited epidemiological evidence linking pesticide exposure to infant leukaemia or suggesting that pesticide exposure may have a greater impact on children than adults, though almost all of the available evidence does not make a distinction between infant and childhood leukaemia. Moreover, most epidemiological studies are limited because no specific pesticides have been directly associated with the risk of leukaemia in children, but rather the broad term “pesticide exposure” (Hernandez and Menendez, 2016). A better characterisation of exposure is needed, particularly to identify the individual chemical (or chemical mixtures) involved, the level of exposure and the specific time window of exposure (whether preconceptional, gestational, neonatal, etc.). In this perspective, the AOP described herein would provide a relevant scientific and regulatory support for understanding the potential of a chemical to be involved in this toxicological pathway.

### **Regulatory relevance of the AOP**

#### *Limitations of the current regulatory guidelines regarding infant leukaemia*

Genotoxicity in general (and as a result of topo II poisoning in particular) and carcinogenicity are apical endpoints in established regulatory guideline studies. Topo II poisoning is one of the potential mechanisms of genotoxicity and carcinogenicity in various International Conferences on Harmonisation (ICH) guidelines for human medicines, for example, ICH M7. It is also known that

some manifestations of genotoxicity in tests measuring chromosomal aberrations, micronuclei or chromosome damage (e.g., Comet assay) are partially due to DSBs created likely by aberrant topo II activity.

Regarding *in vitro* genotoxicity testing, primary cells or permanent cell lines routinely used for regulatory purposes may not be representative of cells in developing organisms in terms of sensitivity and specificity. Very early HSPCs have been considered more sensitive to genotoxicity because of the unfolded nature of their DNA, immature repair systems, high division rate, high topo II expression and activity level. Some *in vivo* tests, for example, the chromosomal aberration test and the micronucleus test, have shown a poor sensitivity, likely because of the low exposure of haematopoietic cells in *in vivo* situations. The only test guideline that covers the developmental period of susceptibility is the extended one-generation test (OECD TG 443), which includes a developmental immunotoxicity cohort. At present, this cohort may identify postnatal effects of prenatal and neonatal exposures since haematology parameters and histopathology of haematopoietic organs are investigated. However, TG 443 has a number of limitations, including a lack of specificity to identify a pattern relevant to infant leukaemia in humans, and a low power due to the reduced number of animals examined.

Regarding the carcinogenicity assay (OECD TG451 and TG453), no treatment is administered during the early *in utero* development phase, the relevant window of exposure for infant leukaemia. Furthermore, it should be noted that these *in vivo* regulatory studies, at their best, are designed to explore specific hazards (e.g., chemically-induced carcinogenesis), but not to explore complex and rare human diseases for which a more comprehensive scientific understanding is required for the identification of potential environmental hazards. Therefore, current regulatory studies have scientific limitations and experimental gaps which might lead to false negative results, and are thus in need of being improved for a more accurate hazard assessment.

Advances in the understanding of physiological processes and mechanisms underlying diseases have not been reflected in the modification of test guidelines required for regulatory decision making on the approval of chemical substances. Moreover, traditional toxicology protocols do not incorporate data generated from emerging technologies pertaining to molecular mechanisms and signalling pathways. Since regulatory decisions should be based on the best available science, more predictive, reliable, faster and less expensive approaches are needed to provide mechanism-based, chemical-specific toxicity information to inform more reliably human health risk assessment.

In Fig 3, we have outlined a larger framework—an Integrated Approach to Testing and Assessment (IATA)—into which the current AOP would fit as an integral part (Tollefsen et al., 2014). If the existing information, also including experimental research within the IATA framework, allows the regulatory decision to be made, there is no urgent need to perform additional experiments. However, if the weight of evidence assessment indicates that the information is inadequate for decision making, generation of new data based on the AOP assays is seen as advisable, followed by a new round of weight of evidence assessment. In this way, only targeted studies could be performed and evaluated under the IATA framework. The proposed IATA would rely not solely on data from regulatory and/or experimental animal studies, but rather on mechanistic understanding and on specific problem formulation. Accordingly, regulatory decision-making would also take into account other data, such as *in silico*, *in chemico* and *in vitro* data, to complement the traditional regulatory data, and the decision making would be tailored to the problem formulation and allowing data gaps to be identified. To help the regulatory scientist in building confidence in data from such tools and their associated predictive models, these new data need to be characterised and qualified. A systematic evaluation of the AOP as well as AOP-informed IATA according to Patlewitz et al. (2015) is recommended.

## CONCLUSIONS

The AOP described herein was developed with the intention to use an epidemiologically-based human health outcome as AO, and then build back an AOP leading to this AO. Infant leukaemia is a human disease and consequently apical regulatory endpoints explored by the experimental toxicological studies, can at their best explore the hazard only by means of surrogate testing. These include genotoxicity and carcinogenesis assays and blood cell analyses in the *in vivo* toxicology assessment. Considering the unique biology of this AO, these tests present some scientific and technical limitations as well as a lack of sufficient sensitivity and specificity for the AO. Additionally, experimental animal models replicating the AO are limited. Technical limitations of the current regulatory tests include the following: standard carcinogenesis studies do not include an early *in utero* exposure time window, blood cell analysis is not a standard requirement in the extended one-generation reproductive toxicity study, and no cancer-related endpoints are included in this study. In addition, considering the rarity and the complexity of the disease, the sensitivity and specificity of the regulatory tests to capture this hazard is likely to be a big hurdle, such that they are unlikely to represent the best way to explore this AO. In addition, some evidence indicates

that the current genotoxicity testing battery lacks sufficient sensitivity and specificity to capture the essential KE, *MLL*-r, and comparative testing for chromosome translocation/aberration between standard cell systems and HSPCs should be a matter of additional research. The AOP described herein clearly indicates that the MIE and the KE1 can be measured in scientific and/or regulatory validated test assays.

With the above premises, the authors support the use of this AOP during the process of assessment of epidemiological studies and the utilisation of the AOP framework to support the biological plausibility of the effects observed in the epidemiological studies when experimental and toxicological studies indicate that the AOP is affected. The AOP conceptual framework should also inform IATA as part of a compound specific risk assessment and guide on which additional studies should be possibly performed, if the case, to fulfil data gaps and integrate the AOP into the mode of action (MOA) framework.

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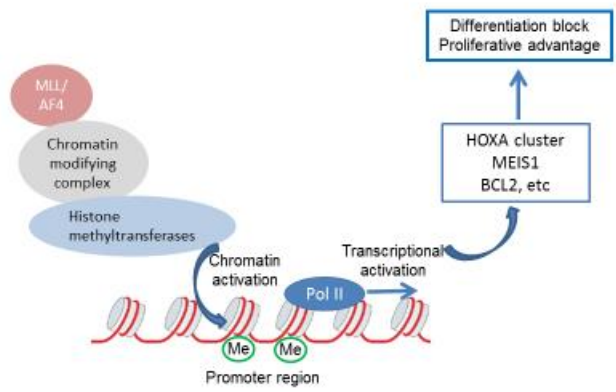
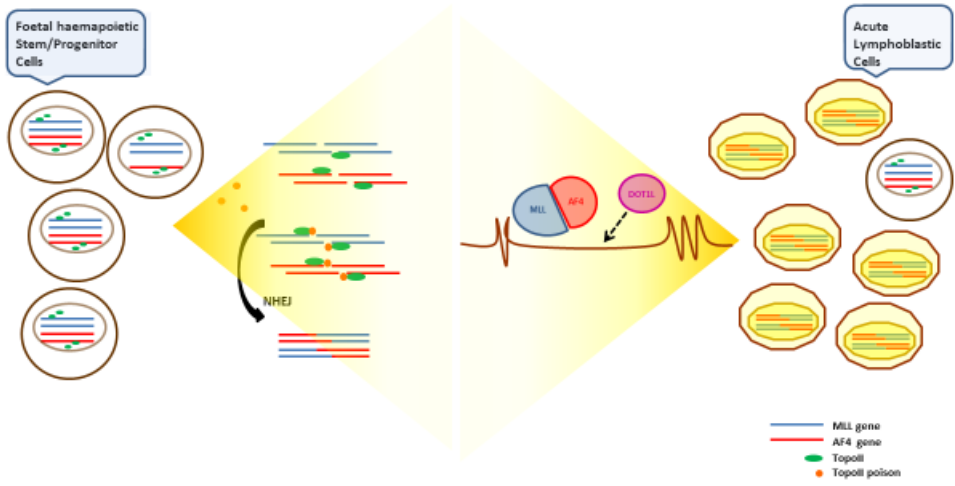
## LEGENDS TO THE FIGURES

**Fig 1** The developed scheme for the Adverse Outcome Pathway (AOP): *In utero* DNA topoisomerase II poisoning leading to infant leukaemia.

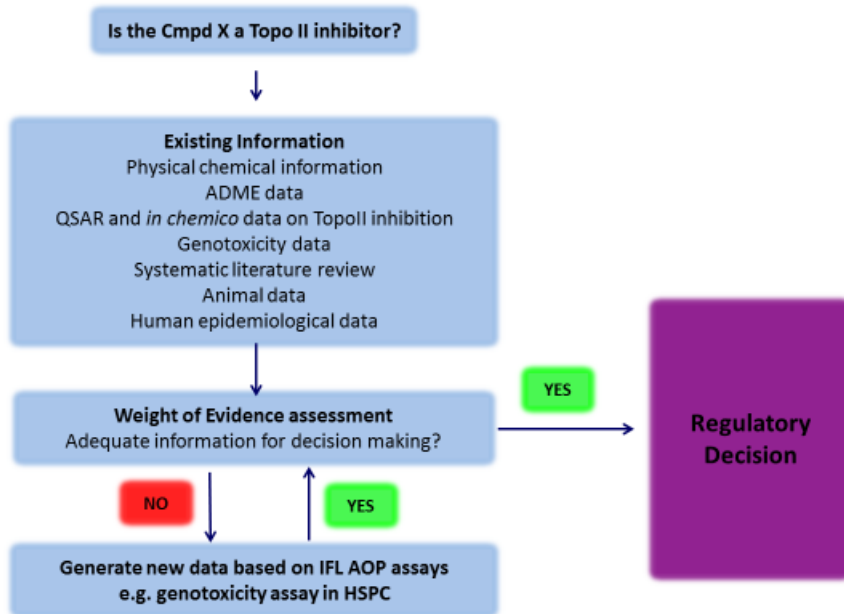
**Fig 2** A schematic view of molecular and biochemical processes distal from MLL fusion gene formation. MLL-fusion protein-containing chromatin modifying complex binds histone methyltransferases, which through histone methylation activate various target genes (HOXA cluster, MEIS1, BCL-2, etc), which cause differentiation block, maintain survival and provide proliferative advantage for MLL-rearranged leukaemia cells (see Marschalek, 2010, Ballabio and Milne, 2012, 2014; Sanjuan-Pla et al., 2015; Benito et al., Cell Rep 2015). It is probable that other fusion proteins activate an analogous chain of events, which leads to similar outcomes.

**Fig 3** Example of the AOP informed IATA for Infant Leukaemia. The goal is to take into consideration all the available information and continue along the weight of evidence assessment for the need of generating new information based on the infant leukaemia AOP assays.

**Adverse Outcome Pathway (AOP):**  
*In utero* DNA Topoisomerase II Inhibition Leading to Infant Leukemia



## IATA Framework for assessing Topo II inhibitors





**Table 1.** Comparison of some characteristics of infant, childhood and treatment-related adult acute leukaemia (see reviews of Cowell and Austin, 2012; Ezoe, 2012; Pendleton and Osheroﬀ, 2014; Sanjuan-Pla et al., 2015; Gole and Wiesmuller, 2015)

<b>Characteristic</b>	<b>Infant leukaemia (esp. MLL-AF4)</b>	<b>Childhood leukaemia</b>	<b>Treatment (etoposide)-related acute leukaemia</b>
Incidence (cases per 10 <sup>6</sup> newborns)	1?	40?	<10 % of the treated patients on particular anticancer regimens (see table 2)
Incidence peak (years of age)	<1	3–5	within 2 years after the onset of treatment
First “hit”	in utero	in utero?	soon after the onset of treatment
Second “hit”	probably not needed	in utero/postnatal	probably not needed
Latency	short	long	short
Prognosis (5-yr)	poor (38%)	good (80%)	poor
Principal rearrangement	MLL-AF4 (about 60–80% of ALL)	TEL-AML1 HYPERDIPLOID BCR-ABL E2A-PBX1 (MLL-AF4 3%)	MLL-AF4 (about 40% of t-AL), PML-RARA in acute pro-myelocytic leukaemia by esp. mitoxantrone
Distribution of break sites at bcr <sup>a</sup>	telomeric	centromeric and telomeric	telomeric
Phenotypic characteristics	Pro-B/mixed: lymphoid CD34+, CD19+, CD10-; myeloid CD15+, CD65+ and NG2+	variable; mostly B-ALL but also AML and T-lymphoid-ALL	Mostly myeloid

<sup>a</sup>bcr, breakpoint cluster region of the *MLL* gene (between exon 8 and intron 10/exon 11)

**Table 2.** The most frequent partners in the infant leukaemia-associated MLL gene rearrangement products (Marschalek, 2010)

<b>Fusion partner</b>	<b>Structural features</b>	<b>Functional features</b>
MLL (KMT2A)	lysine methyltransferase 2A PHD finger protein zinc finger CXXC-type	MLL complex: chromatin activation, cell cycle control, DNA repair MLL•AF4 leukaemogenic
AF4 (AFF1)	super elongation complex	AF4•MLL leukaemogenic
AF6 (AFDN, MLLT4)	adherens junction formation factor, PDZ domain protein	?
AF9 (MLLT3)	super elongation complex subunit	?
AF10 (MLLT10)	PHD finger protein	?
ENL (MLLT1)	super elongation complex subunit	?

**Table 3.** Chemicals identified as DNATopoisoemrase II poisons

Chemical class and chemicals	Positive findings <i>in vitro</i> and <i>in vivo</i>	References (and remarks)
<b>Anticancer agents*</b> (see Cowell and Austin, 2012)		
<i>Epipodophyllotoxins</i> <b>etoposide</b>  teniposide	Topo II poison <i>in vitro</i> , leukaemogenic <i>in vivo</i> in humans (therapy-associated AL) by analogous mechanism of action	Ezoe, 2012; Montecuccio et al., 2015 <b>'Tool chemical' for developing the AOP</b>  in combination treatment regimens
<i>Anthracyclines</i> doxorubicin	MLL breakpoint detection (Thys et al 2015)	Cowell and Austin, 2012 in combination treatment regimens
<b>Bioflavonoids</b> (see Ketron and Osheroff 2014)		
<i>Flavones</i> luteolin, apigenin, diosmetin	Topo II DNA cleavage and MLL rearrangement <i>in vitro</i>	Strick et al., 2000; Bandele and Osheroff, 2007
<i>Flavonols</i> myricetin, quercetin, kaempferol, fisetin	Topo II DNA cleavage and MLL rearrangement <i>in vitro</i> and <i>in vivo</i>	Strick et al., 2000; Bandele and Osheroff, 2007; Vanhees et al., 2011
<i>Isoflavones</i> Genistein (daidzein, biochanin A inactive)	Topo II DNA cleavage and MLL rearrangement <i>in vitro</i> and <i>in vivo</i>	Strick et al., 2000; Bandele and Osheroff, 2007; Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007; Azarova et al., 2010; Vanhees et al., 2011
<i>Catechins**</i>	Topo II cleavage	Austin et al., 1992; Bandele et al., 2008

<i>Isothiocyanates</i> ***	Topo II cleavage	Lin et al., 2014
Curcumin	Topo II cleavage and MLL rearrangements	Lopez-Lazaro et al., 2007; Ketron et al., 2013
<b>Environmental chemicals</b>		
Benzene	MLL breakpoint detection	Thys et al., 2015
Diethylnitrosamine	MLL breakpoint detection	Thys et al., 2015
Chlorpyrifos	MLL rearrangements in human cells	Lu et al., 2015
Permethrin	MLL cleavage in cell culture	Borkhardt et al., 2003

\*anthracyclines (doxorubicin, epirubicin, daunorubicin, idarubicin, aclarubicin), anthracenediones (mitoxantrone) and acridines (amsacrine) differ from epipodophyllotoxins in that in addition to topo II poisoning, they have other mechanisms of action leading to cytotoxicity (Cowell and Austin, 2012). Except for doxorubicin and mitoxantrone, other drugs in these classes have not been specifically studied regarding MLL rearrangements.

\*\*for example EGCG (epigallocatechin gallate) and EGC (epigallocatechin) are very strong topo II poisons *in vitro*, but ECG (epicatechin gallate) and (EC (epicatechin) inactive) are virtually inactive.

\*\*\*benzyl-isothiocyanate, phenethyl-isothiocyanate, sulforaphane.