AOP ID and Title:

SNAPSHOT

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AOP 202: In-utero DNA topoisomerase II poisons leading to infant leukaemia

Short Title: topoisomerase II poisons, infant leukaemia

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Status

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Abstract

Infant leukaemia is a rare haematological disease (1 in 10^6 newborns, accounting for 10% of all childhood acute lymphoblastic leukaemias (ALL)) manifesting soon after birth (<1 year) and having a poor prognosis (Sanjuan-Pla et al 2015). Compared to the more frequent childhood leukaemia, infant leukaemia show distinct features:

- An early neonatal onset linked to its plausible origin as a ‘intrauterine developmental disease’ (Greaves 2015; Sanjuan-Pla et al 2015);
- Rearrangements of the mixed-lineage leukaemia (*MLL; KMT2A*) 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; for infant ALL, about 60-80% carry an MLL rearrangement (Sam et al.2012; Jansen et al.2007) and the percentage for infant acute myeloid leukaemia (AML) is about 40 %;
- The MLL rearrangement at an early stage of development; the likely target cells (still unidentified) are the hematopoietic stem and progenitor cells (HSPC) in fetal liver and/or earlier (mesenchymal) stem cells in embryonic mesoderm (Bueno et al 2009; Menendez et al 2009);
- The infant MLL-rearranged leukaemia carries less somatic mutations (1.3 vs 6.5/case) than the childhood

disease (Andersson et al 2015; Dobbins et al 2013), pointing to the lack of a “second hit” and suggesting a “one big hit” origin.

Overall, based on the available evidence, infant leukaemia pathogenesis originates from a single, severe hit to a target cell during early intrauterine development. Whereas the limited epidemiological studies do not allow any firm conclusion on a possible role for chemicals in infant leukaemia (Pombo-de-Oliveira et al 2006; Ferreira et al 2013), exposures to chemicals able to induce MLL rearrangements through topoisomerase II (TopoII) “poison”, particularly etoposide and other TopoII “poisons”, including some bioflavonoids, have been suggested as agents promoting the driver genetic oncogenic event. Experimental models for infant leukaemia have been developed, but a wholly satisfactory model reproducing the phenotype and latency is not yet available.

Nevertheless, the anticancer drug etoposide can be considered as a model chemical for DNA topoisomerase “poison”. Acute leukaemia is an adverse effect recorded in etoposide-treated patients, showing MLL rearrangements that are in many ways analogous to those in infant leukaemia (Bueno et al 2009; Joannides et al 2010, 2011). Therefore the proposed AOP is supported by a number of convincing inferential evidences by means of using etoposide as a tool compound to empirically support the linkage between the proposed molecular initiating event (MIE) and the adverse outcome (AO). In the meanwhile, this AOP identifies several knowledge gaps, the main ones being the identification of the initiating cell and the investigation of TopoII poisons in a robust model; thus, the present AOP may be modified in future on the basis of new evidence.

Background

Infant leukaemia (<1 year old) is a rare disease of developmental origin distinct from adult and childhood leukaemias which fit the classical two-hit cancer model. Both genetic and haematological studies strongly indicate an *in utero* origin at an early phase of foetal development. Rearrangements of the mixed lineage leukemia (MLL) gene producing abnormal fusion protein are the most frequent genetic/molecular hallmarks in infant B-cell ALL. In small epidemiological studies, mother/foetus pesticide exposure has been associated with infant leukaemia; however, strength of evidence and power of these studies are weak at best. Despite recent advances in the pathogenesis of pediatric leukemia, surrogate models such as *in vitro*, *ex vivo* or animals *in vivo* do not reproduce the human disease sufficiently and they suffer from difficulties in interpretation and extrapolation of findings and from the intrinsic limitation in cancer bio-assay design to cover relevant window of exposure. This adverse outcome pathway (AOP) is based substantially on an analogous disease – secondary acute leukaemia caused by etoposide, a topoisomerase II (TopoII) poison –, and on cellular and animal models. The hallmark of the AOP is the formation of *MLL* gene rearrangements (MLLr) via TopoII poisoning, leading to fusion genes and eventually acute leukaemia by global (epi)genetic dysregulation. The AOP condenses molecular, pathological, regulatory, clinical and epidemiological knowledge in a pragmatic framework with the aspiration of focussing on human specific hazard in the risk assessment process. The AOP enables to identify important gaps of knowledge relevant to 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 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 and represents a transparent and weight of evidence based tool to define the plausible causative mechanism necessary for the interpretation and integration of epidemiological studies in the process of risk assessment.

Summary of the AOP

Stressors

Name	Evidence
Etoposide	Strong

Etoposide

Much of the relevant, albeit indirect, evidence to support this AOP come from the studies on etoposide, an anticancer drug TopoII “poison”, which is known to induce therapy-associated acute leukaemia (t-AL) in adults (Cowell and Austin 2012; Pendleton et al 2014). It is of interest that the latency of t-AL is <2 years between the treatment of the primary malignancy and the clinical diagnosis of the secondary disease and that the prognosis of t-AL is poor (Pendleton et al 2014). t-AL is characterized by the MLL rearrangements and it is practically certain that these fusion genes are caused by etoposide or anthracyclines treatment, because MLL rearrangements have not been detected in bone marrow samples banked before the start of the treatment of the first malignancy. Also the breakpoints in MLL or partner genes fall within a few base pairs of a drug-induced enzyme-mediated DNA cleavage site (Pendleton et al 2014).

Etoposide can induce MLL rearrangements in different cell types; interestingly, embryonic stem cells and their hematopoietic derivatives are much more sensitive than cord blood-derived CD34+ cells to etoposide induced MLL rearrangements; in addition, undifferentiated human embryonic stem cells (hESCs) were concurrently liable to acute cell death (Bueno et al., 2009). These findings suggest that the MIE should be put into evidence in target cell models with appropriate sensitivity.

Molecular Initiating Event

Title	Short name
In utero exposure to DNA topoisomerase II “poisons”	In utero exposure to DNA topoisomerase II “poisons”

1252: In utero exposure to DNA topoisomerase II “poisons”

Short Name: In utero exposure to DNA topoisomerase II “poisons”

AOPs Including This Key Event

AOP ID and Name	Event Type
202: In-utero DNA topoisomerase II poisons leading to infant leukaemia	MolecularInitiatingEvent

Evidence for Perturbation of this Molecular Initiating Event by Stressor

A number of drugs, environmental chemicals and natural substances are identified as TopoII “poisons” (Pendleton et al 2014) (Table 21). A well investigated example is the anticancer drug etoposide; also bioflavonoids, e.g. genistein, (Barjesteh van Waalwijk van Doorn-Khosrovani et al 2007; Azarova et al 2010) bind to TopoII enzymes, induce cleavage in the MLL gene and produce a fusion gene (and its product) in human cells. The organophosphate pesticide chlorpyrifos has been shown to inhibit (‘poison’) the enzyme *in vitro* (Lu et al 2015).

Chemical class	Examples	References
Anticancer agents		
Epipodophyllotoxins	**etoposide**, teniposide	Montecucco et al 2015
Anthracyclines	doxorubicin, epirubicin, daunorubicin, idarubicin, aclarubicin	Cowell and Austin 2012

Anthacenedione	Mitoxantrone	Cowell and Austin 2012
Acridines	Amsacrine	Cowell and Austin 2012
Bioflavonoids		
Flavones	luteolin, apigenin, diosmetin	Ketron and Osheroff 2014
Flavonols	myricetin, quercetin, kaempferol, fisetin	Ketron and Osheroff 2014
Isoflavones	Genistein	Ketron and Osheroff 2014
Catechins	EGCG, ECG, EGC, EC	Ketron and Osheroff 2014
Isothiocyanates	benzyl-isothiocyanate, phenethyl-isothiocyanate, sulforaphane	Ketron and Osheroff 2014
Other phytochemicals	Curcumin	Ketron and Osheroff 2014
Environmental chemicals		
Aromatic compounds	benzene, PAHs	
Nitrosamines	Diethylnitrosamine	Thys et al 2015
Organophosphates	Chlorpyrifos	Lu et al 2015

Etoposide

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Bioflavonoids

Bioflavonoids are natural polyphenolic compounds in a large variety of plant-derived food items. TopoII-mediated DNA cleavage has been linked to genistein, kaempferol, luteolin, myricetin and apigenin (Strick et al 2000; Bandele and Osheroff 2007; Azarova et al 2010; Lopez-Lazaro et al 2010), although the concentrations in in vitro studies have been quite high. It has also been demonstrated that several bioflavonoids are capable of inducing the cleavage of the MLL gene in human cell lines (Strick et al 2000; van Doorn-Khosrovani et al 2007). The in vitro effects of bioflavonoids suggested a possible link between dietary intake and infant leukemia (e.g., Azarova et al., 2010; Lanoue et al., 2010); however until now, epidemiological evidence existing to support or refute such a hypothesis is based on small studies (Ross et al 1996; Spector et al 2005).

Chlorpyrifos

Chlorpyrifos is a widely used organophosphate insecticide, which has been suspected as a risk factor for infant and childhood leukaemia after the house-hold exposure of pregnant women. According to Lu et al (2015), chlorpyrifos and its metabolite chlorpyrifos oxon exhibit an inhibitory effect on in vitro TopoII activity. Chlorpyrifos causes DNA double strand breaks as measured by the neutral Comet assay and induces MLL gene rearrangements in human fetal liver-derived $CD34^+$ hematopoietic stem cells via TopoII 'poisoning' as detected by the FISH assay and in vitro isolated TopoII inhibition assay, respectively (Lu et al 2015). Chlorpyrifos also stabilizes the TopoII-DNA cleavage complex. Etoposide was used a positive reference compound in these studies and it performed as expected. The lowest concentration of chlorpyrifos used was 1 µM and it gave a statistically significant effect in many in vitro assays. The point of departure of etoposide, which was calculated to be 0.01 to 0.1 µM (Li et al 2014), is at least 10-fold lower than that of chlorpyrifos.

Biological Organization

Level of Biological Organization
Molecular

DNA topoisomerases are ubiquitous enzymes, which control the integrity of double-stranded DNA. They are thus key enzymes at all levels of living organisms. The available evidence suggest that important differences in sensitivity to topoisomerase inhibition might exist among different cell types, depending on the amount of proliferative burden, of the TopoII enzymes and on physiological repair processes. Mesodermal precursor or hematopoietic stem and progenitor cells (HSPCs) are rapidly dividing cells with a high content of TopoII and for these reasons they can be a sensitive target during a critical developmental window (Hernandez and Menendez 2016). In addition, evidence from 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 the foetuses and infants than in adults (Udroiu et al 2016). This is possibly indicating a greater sensitivity to genotoxic insult during development which can be due to the higher proliferation rate and lower ability of DNA repair of the hematopoietic stem cells. However, the role that the different microenvironments (foetal liver, infant bone marrow and adult bone marrow) during ontogenesis can exert on cell sensitivity cannot be ruled out (Udroiu et al. 2016). The existence of relevant interspecies differences is unknown, but it cannot be ruled out presently.

How this Key Event Works

DNA topoisomerase (Top) II enzyme "poisons" disturb the normal TopoII enzyme function and cause a 'hanging double strand break (DSB)' at a specified DNA sequence. The above description of the MIE is of significance because there are 3 different kinds of "poisons" of TopoII enzyme, out of which competitive inhibitors prevent the function of the enzyme and cause cell death, whereas other interfacial and covalent inhibitors may cause – depending on the situation – other consequences of DNA damage response including chromosomal rearrangements (Pendleton et L 2014; Lu et al 2015). A further prerequisite for the specific outcome, i.e. creation of chromosomal rearrangement, is that TopoII "poison" has to occur in an especially vulnerable and correct hot spot in the MLL locus in the right target cell vulnerable to transformation.

TopoII enzymes have several crucial functions in DNA replication, transcription, repair and chromatin remodelling, i.e. TopoII enzymes take care of DNA integrity and topology. Because the enzyme functions by passing an intact double helix through a transient double-stranded break, any disturbances in its function, e.g. by chemical inhibitors, could have a profound effect on

genomic stability, resulting in DNA repair response, gene and chromosomal damage, initiation of apoptosis and ultimate cell death. A double-strand break and error-prone non-homologous end-joining (NHEJ) DNA repair mechanism may lead to gene rearrangements; chromosomal translocations and consequently fusion genes (see Figure 33). A comprehensive description of TopoII enzymes and their functions and derangements could be found in recent review articles (Cowell and Austin 2012; Pendleton et al 2014; Ketron and Osheroff 2014).

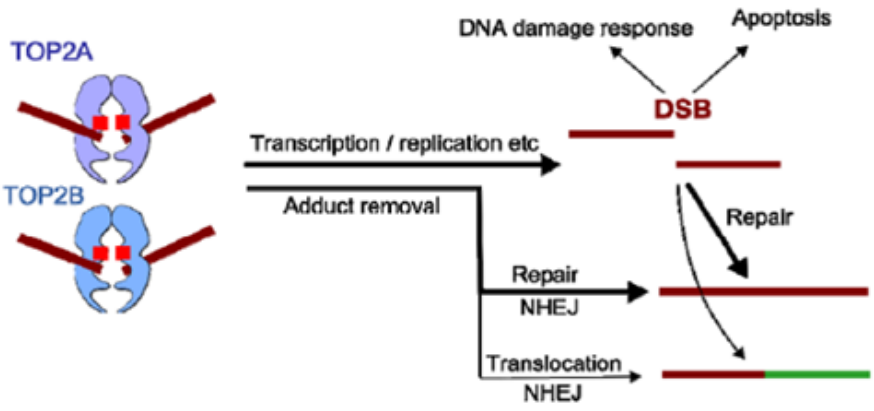

Fig.33: TOP2 Poisons, downstream events. TOP2 poisons inhibit the religation step of the TOP2 reaction cycle, leading to accumulation of covalent TOP2-DNA cleavage complexes. These lesions are cytotoxic and lead to activation of the DNA damage response and potentially apoptosis. Alternatively these lesions are repaired, largely through the non-homologous end-joining pathway. Translocations observed in therapy-related leukemia are presumed to occur as a result of mis-repair, joining two heterologous ends. (from Cowell and Austin 2012)

How it is Measured or Detected

The identification and measurement of the inhibition of TopoII enzymes is made more difficult by the presence of different molecular mechanisms (see above). However, some assays are used in pharmacological research to screen TopoII “poisons”, including cell-free decatenation assay (Schroeter et al., 2015). The most important mode, the cleavage activity of TopoII can be studied in vitro, by using a human recombinant enzyme and an appropriate double-stranded plasmid as a target to quantitate double-strand breaks (Fortune and Osheroff 1998). A cleavage can also be indirectly detected by measuring various indicators of DNA damage response, such as ATM activity, p53 expression, γH2AX or Comet assay (Li et al 2014, Schroeter et al., 2015, Castano et al 2016).

It is useful to note that several chemicals identified as TopoII “poisons”do require metabolic oxidation to become active inhibitors. Etoposide itself is converted via the catechol metabolite to etoposide 3-quinone, which is a covalent TopoII poison (Smith et al 2014), whereas etoposide and its catechol are interfacial inhibitors. Curcumin is also an active TopoII poison due to its oxidized metabolites (Gordon et al 2015). This fact deserves consideration if a screening for TopoII inhibition is envisaged.

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Key Events

Title	Short name
In utero MLL chromosomal translocation	MLL translocation

1253: In utero MLL chromosomal translocation

Short Name: MLL translocation

AOPs Including This Key Event

AOP ID and Name	Event Type
202: In-utero DNA topoisomerase II poisons leading to infant leukaemia	KeyEvent

Stressors

Name
Etoposide

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Although the KE deals with the general process of DNA integrity, the available evidence do not allow for evaluating

whether any significant difference occurs among cell types or species. It has been shown that the mouse has an analogous fusion gene *mll-af4*. A recent study has shown that in utero exposure to etoposide induces *mll* translocations in Atm-knockout mice, which are defective in the DNA damage response, albeit not in wild-type mice; moreover, fetal liver hematopoietic stem cells were more susceptible to etoposide than maternal bone marrow mononuclear cells, pointing out the life stage-related susceptibility in regards to TopoII “poison” also in the mouse (Nanya et al., 2015).

MLL-AF4 fusion gene is present and expressed in bone marrow mesenchymal stem cells in infant patients with t(4;11) B cell-ALL (Menendez et al. 2009). 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 et al. 2015; sanjuan-Pla et al. 2015).

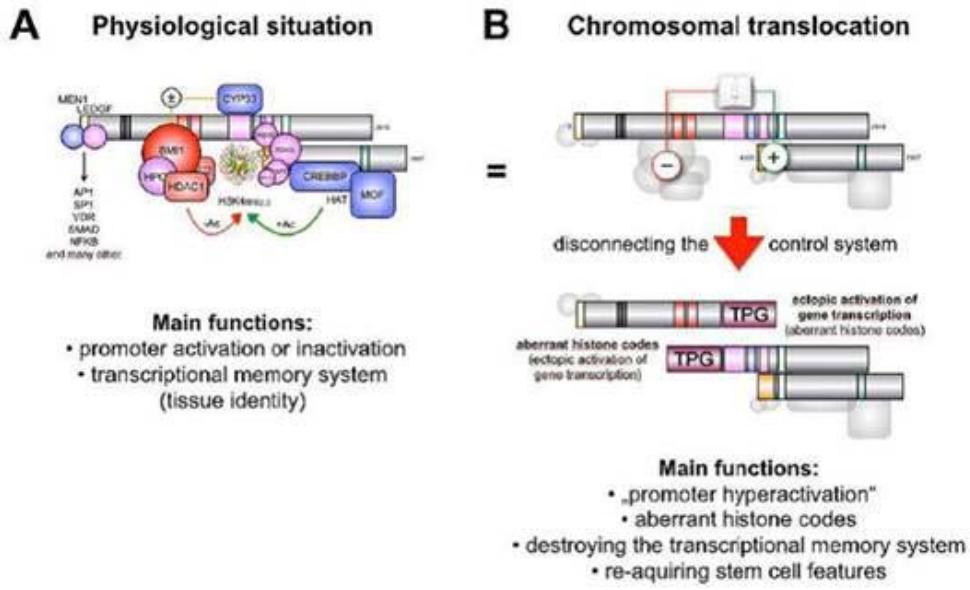
How this Key Event Works

Chromosomal rearrangements of the mixed-lineage leukaemia (MLL) gene, located on the q23 band of chromosome 11 (11q23), are the genetic hallmark of most infant leukaemias (Meyer et al 2013; Sanjuan-Pla et al 2015). MLL is located within the fragile site FRA11G; chromosomal fragile sites are regions of the genome susceptible to breakage under conditions of replication stress; interference with TopoII may promote fragile site instability. MLL encodes a protein homologous to the Drosophila trithorax gene, which has relevant functions in embryogenesis and hematopoiesis (Ernest et al 2004, Hess et al 1997).

There are many translocation and fusion partners for MLL; DNA breakage within MLL can lead to rearrangement with over 120 partner genes (Meyer et al 2013). In principle all MLL fusion genes are potential initiating drivers, although clinical studies have shown a 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 predominant 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 a manifest disease resulting from this rearrangement in *in vivo* animal models. For AML, about 30 % of the patients carry an MLL rearrangement.

The occurrence of MLL rearrangements at a very early fetal development is highly probable on the basis of neonatal blood spot analysis and by the high concordance rate of infant leukaemia in monozygotic twins (Ford et al 1993; Gale et al 1997; Sanjuan-Pla 2015). Menendez et al (2009) showed that MLL-AF4 fusion gene is present in bone marrow mesenchymal stem cells in infant leukaemia patients, but not in patients of childhood leukaemia, suggesting that the origin of the fusion gene is probably prehaematopoietic. Consequently, the affected cell, the so called leukaemia-initiating cell, may be an early prehaematopoietic mesodermal precursor, a hematopoietic stem cell or hematopoietic progenitor cell residing mainly in the liver (Greaves 2015; Sanjuan-Pla et al 2015).

MLL protein (complexed with a large number of other protein factors) serves as a transcriptional activator or repressor via the binding to promoter regions of active genes, marking these regions by covalent histone modifications (Sanjuan-Pla et al 2015). Translocation and creation of fusion genes and products destroys the intrinsic control mechanisms of the MLL protein. The resulting ‘ectopic’ functions involve promoter hyper-activation and re-acquiring stem cell features (Sanjuan-Pla et al 2015). A schematic presentation of the drastic changes of the MLL product is depicted in the figure below.



Proposed model for the oncogenic conversion of MLL fusion: A. Physiological situation and B: . A chromosomal translocation, which leads to the intrinsic regulatory mechanism of MLL being destroyed. (Sanjuan-Pla et al. 2015).

MLL translocation sites (breakpoint sequences) in the therapy-related leukaemia fall within a few base pairs of etoposide-induced enzyme-mediated DNA cleavage site (r). Although rearrangements associated with infant leukaemias are often more complex than those observed in treatment-related leukaemias, many are nevertheless associated with stable TopoII-mediated DNA cut sites. Although all these findings are indirect regarding infant leukaemia, they are nevertheless rather persuasive in this respect.

Growing 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 (and other partner) fusion gene and product in causing pleiotropic effects in the affected cell and directing it to the obligatory pathway to the adverse outcome of leukaemia (see KER2).

How it is Measured or Detected

The presence and structure of a fusion gene can be identified with PCR or related techniques. Mapping of cleavage sites in the gene needs genomic DNA. In cells or tissues, the detection of a fusion gene is possible by appropriate immunofluorescent techniques.

Assays measuring chromosomal aberrations, micronuclei or DNA and chromosome damage (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

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Adverse Outcomes

Title	Short name
Infant leukaemia	IFL

1254: Infant leukaemia

Short Name: IFL

AOPs Including This Key Event

AOP ID and Name	Event Type
202: In-utero DNA topoisomerase II poisons leading to infant leukaemia	AdverseOutcome

Stressors

Name
Etoposide

Etoposide

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 $CD34^+$ 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 µM (Li et al., 2014). This range is in agreement with the finding that in human foetal liver $CD34^+$ cells an increase in DSBs was observed at a concentration of 0.14 µM (Bueno et al., 2009) and MLL-r were detectable by FISH or flow cytometry at higher concentrations (Moneypenny et al., 2006).

Biological Organization

Level of Biological Organization
Individual

Evidence Supporting Applicability of this Event

Life Stage Applicability

Life Stage	Evidence
Birth to <1 month	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Infant leukaemia is a paediatric leukaemia likely resulting from gene-environmental interactions. The limited data available suggest that dietary and environmental exposure to substances targeting topoisomerases together with reduced ability of the foetus or their mother to detoxify such compounds because of the polymorphic variants of given genes could contribute to the development of this AO (Hernadez et al. 2016).

In animals the disease is not known and artificial animal models able to reproduce the disease have limitations.

Bardini et al (2015) has however developed a xenograft mouse model with patient MLL-AF4-involving leukoblasts transplanted.

How this Key Event Works

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

How it is Measured or Detected

Haematological methods – identification of leukaemia cells and routine blood cell counts; observations of clinical symptoms.

Following clinical diagnosis, methods for refined diagnosis include bone marrow aspirates for immunophenotypic analyses and cytogenetic assays for molecular stratification.

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

Regulatory Examples Using This Adverse Outcome

Genotoxicity in general and carcinogenicity are apical endpoints in established regulatory guideline studies. TopoII poisoning has been listed as one of the potential mechanisms of genotoxicity and carcinogenicity in the ICH M7 guideline for human medicines. It is also known that some manifestations of genotoxicity in tests measuring chromosomal aberrations, micronuclei or DNA and chromosome damage (Comet assay) are partially due to double-strand breaks created by the disturbed action of TopoII enzymes.

The extended one generation test (OECD 443) includes a developmental immunotoxicity cohort. At present the cohort may identify post-natal effects of prenatal and neonatal exposures on the immune tissues and white blood cells population. However, each regulatory guideline study has potential limitations e.g. no specific parameters are in place to identify a pattern relevant to infant leukemia in humans in the extended one generation test, no treatment is occurring during the early in-utero development phase in the carcinogenicity assay and no considerations on the possible higher sensitivity of the HSC are in place for the genotoxicity assays.

Epidemiological evidence linking pesticide exposure to infant leukaemia, also suggests 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. However, most epidemiological studies are limited because no specific pesticides have been directly associated with the risk of leukaemia, but rather the broad term “pesticide exposure” (Hernandez and Menendez 2016). In this perspective, this AOP would provide a regulatory relevant support for understanding the potential of a chemical to be involved in this toxicological pathway.

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Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
In utero exposure to DNA topoisomerase II “poisons”	directly leads to	In utero MLL chromosomal translocation	Strong	Not Specified
In utero MLL chromosomal translocation	directly leads to	Infant leukaemia	Strong	Not Specified

In utero exposure to DNA topoisomerase II “poisons” leads to MLL translocation

Evidence Supporting Applicability of this Relationship

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

DNA topoisomerases are ubiquitous enzymes, which control the integrity of double-stranded DNA. They are thus key enzymes at all levels of living organisms. The available evidence suggest that important differences in sensitivity to topoisomerase inhibition might exist among different cell types, depending on the amount of proliferative burden, of the TopoII enzymes and on physiological repair processes. Mesodermal precursor or hematopoietic stem and progenitor cells (HSPCs) are rapidly dividing cells with a high content of TopoII and for these reasons they can be a sensitive target during a critical developmental window (Hernandez and Menendez 2016). In addition, evidence

from 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 the foetuses and infants than in adults (Udroiu et al 2016). This is possibly indicating a greater sensitivity to genotoxic insult during development which can be due to the higher proliferation rate and lower ability of DNA repair of the hematopoietic stem cells. However, the role that the different microenvironments (foetal liver, infant bone marrow and adult bone marrow) during ontogenesis can exert on cell sensitivity cannot be ruled out (Udroiu et al. 2016). The existence of relevant interspecies differences is unknown, but it cannot be ruled out presently.

How Does This Key Event Relationship Work

Certain TopoII poisons stabilize the intermediate cleavage complex and prevent the religation with appropriate DNA strands. Covalently DNA end-bound TopoII protein is digested and a hanging end is created. The same process happens in the translocation partner gene. Hanging ends of both genes are processed and subsequently joined by non-homologous end joining (Cowell and Austin 2012). There is evidence that this inappropriate joining of ‘hanging ends’ happens in the same transcriptional factory (hub), and the result is a fusion gene and ultimately 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 that has been a result of a very early chromosomal translocation and rejoining. It is of interest that the simultaneously induced specific DSBs in 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).

Weight of Evidence

Biological Plausibility

The KER as such is biologically plausible. Type II topoisomerases are ubiquitous enzymes which are essential for a number of fundamental DNA processes. As they generate DNA strand breaks, they can potentially fragment the genome. Indeed, while these enzymes are essential for the survival of proliferating cells they can also have significant genotoxic effects by means of accumulation of DNA strand breaks that, if not resulting in cell death may lead to chromosomal translocation in the surviving cell population (McClendon et al. 2007). DNA breaks and MLL rearrangements by etoposide and bioflavonoids have been demonstrated in human fetal liver haematopoietic stem cells, in human embryonic stem cells and in human prehaematopoietic mesenchymal stem cells as well as in cord blood mononuclear cells (Ishii et al 2002; Blanco et al 2004; Moneypenny et al 2006; Bueno et al 2009; Menendez et al 2009), which clearly shows that TopoII-associated MLL rearrangements are produced in appropriate human cells in utero.

Empirical Support for Linkage

There are animal models for infant leukaemia which recapitulate at least some salient aspects of the disease (Sanjuan-Pla et al 2015). However, for example the MLL-AF4 knock-in mice develop leukaemia only after a prolonged latency (Chen et al 2006), thus not recapitulating the ‘pathognomonic’ feature of infant leukaemia.

Etoposide treatment in vivo in mice at day 13.5 of pregnancy induces MLL breakage in fetal liver haematopoietic stem cells in utero, but MLL-rearranged fusion mRNAs were detected only in mice which were defective in the DNA damage response, i.e. atm knockout mice. A fusion gene analogous to MLL-AF4 was not detectable in the wild type mice. In this study, an intraperitoneal injection of 10 mg/kg of etoposide into pregnant mice at day 13.5 of pregnancy resulted in a maximum fetal liver concentration of about 5 µM. A dose of 0.5 mg/kg did not result in a measurable concentration. A statistically significant increase (about 6-fold) in DSBs in the MLL gene of isolated fetal liver haematopoietic stem cells was observed after a single dose of 1 mg/kg to pregnant mice[1]. A clear activation of DNA damage response was observed at the dose of 10 mg/kg (Nanya et al. 2016).

There is a lot of information about the interaction of etoposide with TopoII enzymes and MLL chromosomal translocation at the cell culture level and in connection with treatment-related leukaemia.

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, but when the number of DSBs exceeds a certain value; ATM and p53 become fully activated through reversible mechanism,

leading to elevated repair capacity. The model was able to capture quantitatively 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 µM (Li et al. 2014). This range is in agreement with the finding that in human fetal liver CD34+ cells an increase in DSBs was observed at a concentration of 0.14 µM and MLL translocations were detectable by FISH or flow cytometry at higher concentrations (Moneypenny et al 2006).

[1] Hypothetically, based on linear extrapolation from the dose of 10 mg/kg, the concentration would be of the order of 0.5 µM.

Uncertainties or Inconsistencies

· A prerequisite for the specific outcome, i.e. creation of chromosomal rearrangement, is that TopoII inhibition has to occur in an especially vulnerable and correct hot spot in the MLL locus; however, details of this process and how it happens are not clear.

· A target cell, i.e. leukaemia-initiating cell, has not been identified with sufficient confidence and consequently there is no target cell model to recapitulate the linkage between TopoII inhibition (‘poisoning’) and the production of DSB in an appropriate target. Recently, by the expression of engineered nucleases (TALENs) to induce simultaneous patient specific double strand breaks in the MLL gene and two different known translocation partners (AF4 and AF9), Breese et al (2015) were able to produce specific chromosomal translocations in K562 cells and in primary HSPCs.

· In-utero etoposide-treatment failed to induce leukaemogenesis (Nanya et al 2015). Consequently, the envisaged linkage has not been empirically supported or rejected. However, it should be kept in mind that, whereas etoposide does induce a large number of MLL rearrangements, most of them occur within non-coding regions, therefore not eliciting any direct oncogenic consequence. A 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; thus it may be difficult to empirically support this process.

· Dose-response relationships between etoposide and treatment-related leukaemia are difficult to unravel, but risk of leukaemia seems to increase with larger total exposure to etoposide. However, comparison of exposures or kinetics of etoposide between leukaemia patients and non-leukemic treated subjects did not reveal any significant differences (Relling et al 1998). Also, it is not known whether the etoposide (or metabolite) concentrations during the treatment are of significance. In child and adult chemotherapy, concentrations are extremely variable between individuals; the lowest through plasma concentrations of etoposide have been of the order of 1 µM and peak concentrations very much higher. For example, in a study of Relling et al (1998), the maximum plasma concentration of etoposide was about 90 µM and that of etoposide catechol about 100-times less, below 1 µM. In another high dose chemotherapy study (Stremetzne et al 1997), the etoposide concentration was 170 µM and that of the catechol metabolite 5.8 µM maximally. However, it is not straightforward to juxtapose plasma concentrations and the tissue or cell concentration which TopoII enzyme ’sees’. Penetration of etoposide or its metabolite through plasma membrane is probably rather slow and it has been shown that the brain cancer tissue (metastasis or glioma) to plasma ratio for etoposide is only 0.1 (Pitz et al 2011). Blood-brain barrier is not necessarily a good model for cross-membrane distribution, but may give some idea about the general distributional behaviour of a drug. Even if the active target concentration of etoposide is only 10 % of the plasma concentration, it is still in the same range as the effective concentrations in cellular studies (see above). A final note on relevant concentrations: etoposide concentrations resulting in DSB and fusion gene are probably within a relatively restricted range. The concentration resulting in a proper fusion gene should be in a range which gives rise to a partially repaired insult and cells bypassing death and accumulating the abnormality.

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MLL translocation leads to IFL

Evidence Supporting Applicability of this Relationship

Life Stage Applicability

Life Stage	Evidence

Birth to <1 month	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

DNA topoisomerases are ubiquitous enzymes, which control the integrity of double-stranded DNA. They are thus key enzymes at all levels of living organisms. The available evidence suggest that important differences in sensitivity to topoisomerase inhibition might exist among different cell types, depending on the amount of proliferative burden, of the TopoII enzymes and on physiological repair processes. Mesodermal precursor or hematopoietic stem and progenitor cells (HSPCs) are rapidly dividing cells with a high content of TopoII and for these reasons they can be a sensitive target during a critical developmental window (Hernandez and Menendez 2016). In addition, evidence from 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 the foetuses and infants than in adults (Udroiu et al 2016). This is possibly indicating a greater sensitivity to genotoxic insult during development which can be due to the higher proliferation rate and lower ability of DNA repair of the hematopoietic stem cells. However, the role that the different microenvironments (foetal liver, infant bone marrow and adult bone marrow) during ontogenesis can exert on cell sensitivity cannot be ruled out (Udroiu et al. 2016). The existence of relevant interspecies differences is unknown, but it cannot be ruled out presently.

How Does This Key Event Relationship Work

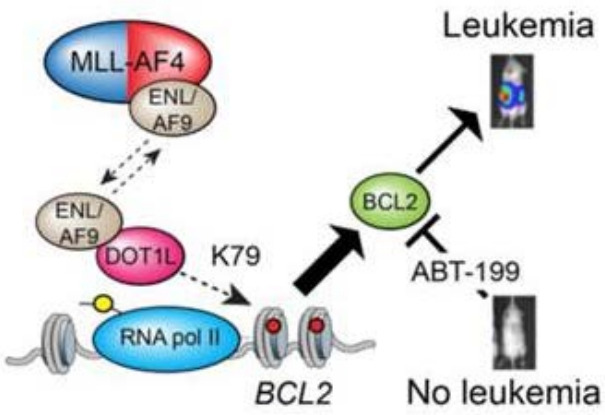
Propagation of a leukaemic cell clone is based on both blockage of differentiation to more mature cells and ability to expand in an uncontrolled way. Formation of the MLL-rearranged fusion genes and their protein products are intimately involved in both the blocked differentiation of HSPCs and the expansion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

Molecular mechanisms

The MLL is the most common translocation gene 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 either AF4, AF6, AF9, AF10, or ENL (Krivtsov and Armstrong 2007). Due to the DNA-binding properties of the N-terminal MLL motif, these fusion proteins are always nuclear and bind to target genes controlled by MLL irrespective of the normal location of the C-terminal partner.

Many fusion proteins have been shown to recruit DOT1L (catalyzing methylation of histone H3K79) to the promoters of MLL target genes and this recruitment seems to be a common feature of many oncogenic MLL fusion proteins. 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 leukemogenesis (Chen and Armstrong 2015). The enzymatic activity of DOT1L is critical to the pathogenesis 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 leukemogenic gene expression, and selectively kills cultured cells bearing MLL translocations (Chen and Armstrong 2015). One of the target gene 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). Expression of BCL-2 is high in human MLL-AF4 leukemia cells from a large number of patients. A specific BCL-2 inhibitor, ABT-199 is capable of killing MLL-AF4 leukaemia cells and prevents cell

proliferation in xenograft mouse leukaemia models (Benito et al 2015). Furthermore, a MLL-AF4 cell line is sensitive to a combination of ABT-199 and DOT1L inhibitors. The figure below provides a schematic representation of the molecular pathway.



MLL-rearranged acute lymphoblestic leukemisa activate BCL-2 through H3K79 methylation and are sensitive to the BCL-2 specific antagonist ABT-199 (benito et al, Cell Rep 2015).

Possible facilitating mutated genes

Recurrent activating mutations in the components of the PI3K-RAS signalling pathway have been detected in almost half of the tested MLL-rearranged ALLs in one study (Andersson et al 2015). Prenatal origin of RAS mutations have been demonstrated also in other studies of infant leukaemia with frequencies of about 15-25 % of cases (Driessen et al 2013; Prelle et al 2013; Emerenciano et al 2015). Emerenciano et al (2015) are of the opinion that RAS mutations seem not to be driver mutations, but may aid disease onset by accelerating the initial expansion of cells.

Overall the activation of the RAS pathway could support the extremely rapid progression of the infant leukaemia. Under this view the mechanism 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 of Prieto et al (2016),the activated K-RAS enhanced extramedullary haematopoiesis of MLL-AF4 expressing cell lines and cord blood-derived CD34+ hematopoietic stem/progenitor cells that was associated with leucocytosis and central nervous system infiltration, both hallmarks of infant MLL-AF4 leukaemia. However, K-RAS activation was insufficient to initiate leukaemia, supporting that the involvement of RAS pathway is an important modifying factor in infant leukemia. It has also been demonstrated that MLL-AF6 fusion product sequesters AF6 into the nucleus to trigger RAS activation in myeloid leukaemia cells and it is possible to attenuate the activation by tipifarnib, a RAS inhibitor (Manara et al 2014).

A possibility that MLL fusions render cells susceptible to additional chromosomal damage upon exposure to etoposide was studied 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). In short, the expression of fusion genes does neither influence DNA signaling nor DNA-DSB repair.

Weight of Evidence

Biological Plausibility

The biological plausibility linking the MLL translocation to infant leukaemia is strong. Rearrangement in the MLL gene is commonly associated with infant acute leukaemia and the disease has unique clinical and biological feature (Ernest et al. 2002). An in utero initiation, an extremely rapid progression, and a silent mutational landscape of infant leukaemia suggest that the MLL-translocation-associated gene fusion product is itself sufficient to spawn leukaemia

and no “second hit” is required. Therapy-related leukaemias following exposure to the topo II poisons such as etoposide are characterized by the MLL chromosomal translocation (Libura et al. 2006, Super et al.1993) and translocations involving MLL are associated with a gain of function and leukemogenic effect (Yu et al. 1998). A critical developmentally early window of stem cell vulnerability, involving perhaps lesions based on epigenetically controlled regulatory factors, has been suggested to explain a rare occurrence and an exceptionally short latency of infant leukaemia (Greaves 2015; Sanjuan-Pla et al 2015). In primary HSPCs genome engineered for patient specific MLL translocations it was possible to show that this specific ‘artificial’ initiation can induce a selective advantage in survival in extended culturing and a higher clonogenic potential in colony forming assay (Breese et al. 2015).

Empirical Support for Linkage

A number of MLL-fusion products, such as MLL-AF9 and MLL-ENL, have shown leukemogenic potential in cord-blood stem cells. Although the MLL rearrangement is essential to develop leukaemia, it alone may not be sufficient and activation of cellular proliferation might be necessary for overt leukaemia (Nanya et al. 2015).

There are several animal models, in which MLL-AF4 fusion gene has been expressed (Chen et al 2006; Metzler et al 006; Krivtsov et al 2008; Bursen et al 2008; Tamai et al 2011). In all these models leukaemia is ultimately developed, but latency has been very protracted. In any case, one could conclude that the expression of the MLL-AF4 fusion gene is capable of developing leukaemia, but it is unknown whether facilitating or necessary changes are required during the long latency in mouse.

Gene engineered human HSPCs carrying MLL rearrangements showed that a subset of cells persisted over time and demonstrated a higher clonogenic potential in colony forming assay (Breese et al. 2015).

Transcription activator-like effector nuclease (TALEN)-mediated genome editing generated 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. The leukemias displayed phenotypic and morphologic similarities with patient leukemia 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.

Uncertainties or Inconsistencies

· The MLL-AF4 knock-in mice develop leukaemia only after a prolonged latency (Chen et al 2006), thus not recapitulating the ‘pathognomonic’ feature of infant leukaemia. Also other animal models have been developed with similar results. Thus, an adequate experimental model for infant leukaemia is still in need.

· The role of a reciprocal fusion gene AF4-MLL in leukemias is controversial: it has a transformation potential in animal model (Bursen et al 2010), but it is not expressed in all MLL-AF4 patients (Andersson et al 2015). The potential role of other reciprocal fusion genes has not been studied.

· Beyond MLL rearrangements, activation of cellular proliferation by mutation or other (epi)genetic insults might be necessary for overt leukaemia. Further studies are necessary to fully understand which factors would contribute to convey a proliferative advantage, as observed in cells with MLL translocation, to leukaemia.

Quantitative Understanding of the Linkage

Relationships between different fusion genes and subsequent leukemia types are incompletely understood. Although roughly 70-80 % of infant B-ALL leukemias carry MLL rearrangements, in 20-30 % of the cases there are no MLL rearrangements. In AML and T-ALL leukemia cases MLL rearrangements are even rarer.

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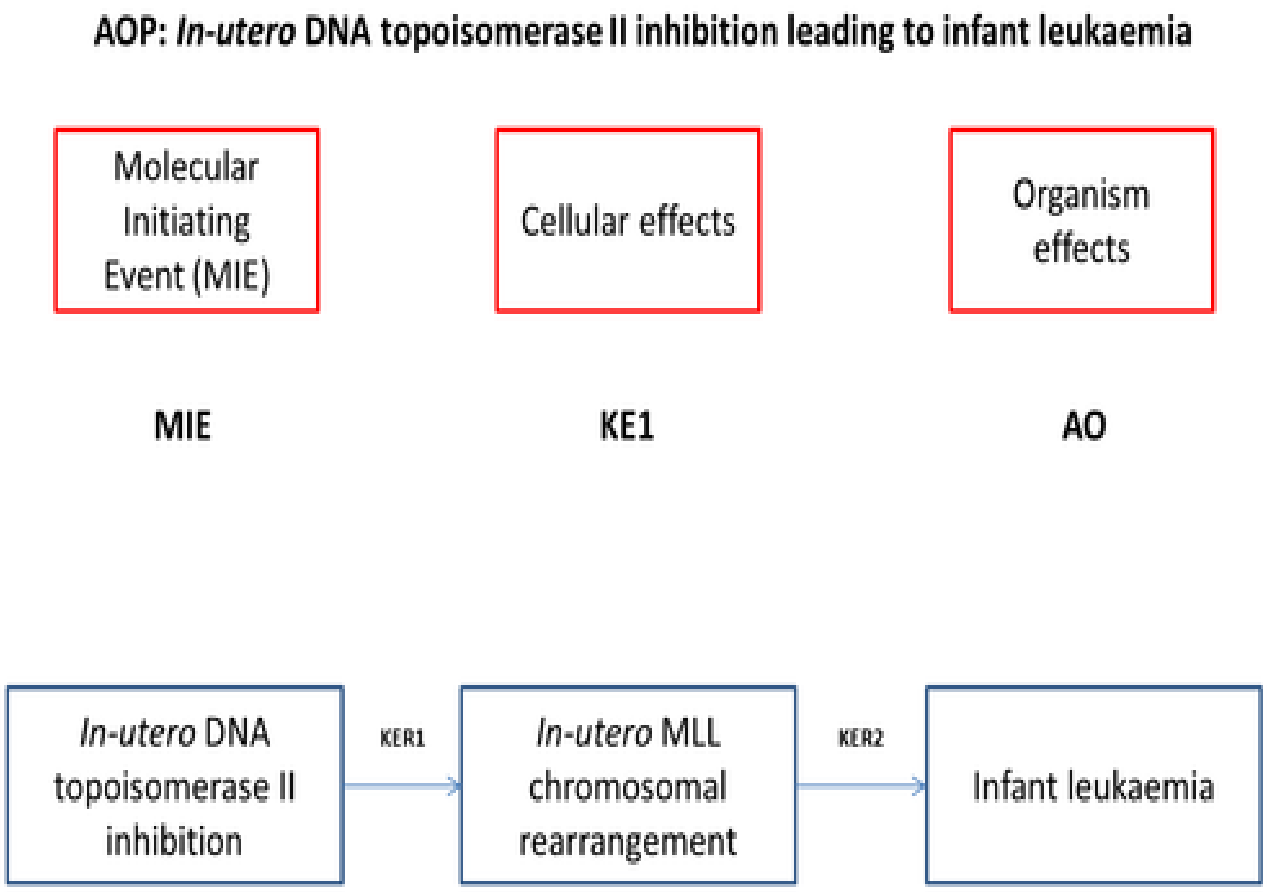
Graphical Representation



Overall Assessment of the AOP

Infant leukaemia is a “hidden” disease quite concretely: initiation occurs in utero at an early phase of foetal development. Studies both in identical twins (Ford et al 1993) and in neonatal blood samples retrospectively (Gale et al 1997) strongly indicate *in utero* origin of the disease. Consequently, direct studies in pregnant humans are difficult or impossible and one has to resort to surrogate in vitro or ex vivo studies or to animal models which necessarily are associated with difficulties in interpretation and extrapolation. Thus, what is described in this overall assessment is based largely on inferences from analogous diseases using tool chemicals able to reproduce the biological basis of the disease (especially etoposide (a Topoisomerases II poison)-caused acute leukaemia in children or adults) or from cellular and animal models.

1. Concordance of dose-response relationship

The only in utero study in mice (Nanya et al 2016) has shown that the dose of 0.5 mg/kg (day 13.5 of pregnancy) does not result in measurable etoposide concentration in foetal liver HSCs whereas the dose of 10 mg/kg leads to a maximal concentration of 5 µM. A statistically significant increase in double strand break (DSBs) in MLL gene was observed at a dose of 1 mg/kg, which would result in a concentration of 0.5 µM by linear extrapolation. In treatment-related acute human leukaemia, various treatment schedules in adults and children give rise to etoposide concentrations between (roughly) <1 µM (through) to >150 µM (peak). There are no adequate experimental systems to study dose-response and response-response relationships across MIE, KEs and AO in a single model.

2. Temporal concordance among the MIE, KEs and AO

There are no serious doubts about temporal concordance among MIE, KEs and AO. It is very difficult to see any other sequence of events (among this AOP), which would bring the AO into effect. Another matter is that it has never been shown in human pregnancy (or will be reliably or robustly demonstrated in the foreseeable future). In this respect, it is difficult to envisage whether epidemiological studies that are possible in humans, would ever be able to demonstrate the link without a direct biomarker for the MIE and KE1. Available experimental models

(Sanjuan-Pla et al 2015) are in conformation with the AOP, except that in experimental in vivo models a very protracted appearance of leukaemia is not in line with a very short latency of infant leukaemia in human.

It is obvious that there exists a vast gap between wide exposure to potential TopII poisons and the rarity of infant leukaemia. On the basis of studies in human adult and childhood leukemias, there are a large number of genetic, epigenetic and host factors potentially modifying the link between topII poisons and leukaemia. Because of the rarity of the disease, it is difficult to envisage an even partial proofing these factors as of importance for the infant leukaemia.

Response-Response and Temporality Concordance for the tool compound etoposide

Concentration of etoposide	MIE In utero DNA topoisomerase II inhibition	KE1 In utero MLL chromosomal rearrangement	AO Infant leukaemia
0.01 – 0.1 µM, *in vitro(TopII enzymes and cells in culture)*	+++ (DNA damage response in various cells)	-	
0.1 – 1 µM, *in vitro cell cultures*	+++ (haematopoietic progenitor and stem cells)	+	
0.5-5 µM, *ex vivo,* mouse fetal liver HSC concentration[1]	+++ (inference from MLL cleavage)	+ (only MLL cleavage)	- (no leukemia development)
max 5 µM, *ex vivo,* mouse fetal liver HSC concentration[1]	+++ (inference from MLL cleavage)	+ MLL fusions detected only in DNA repair deficient mice	- (no leukemia development)
Max >150 µM, plasma concs in etoposide-treated patients[2]	+++ (inference from MLL cleavage)	++ MLL-AF4 fusion gene and protein	+ treatment-related acute leukaemia

[1]a range of concentrations is linearly extrapolated on the basis of the concentration of 5 µM after the dose of 10 mg/kg.

[2]plasma concentration of etoposide cannot be directly extrapolated to the concentration at the active site. Probably the actual active cellular concentrations of etoposide is much lower, perhaps 10 % or less of the plasma concentration.

3. Strength, consistency of the experimental evidence, and specificity of association of AO and MIE

Regarding the treatment-related acute leukaemia, strength, consistency and specificity of association of AO and MIE is strong, because only etoposide and a few other TopII-poison anticancer agents (Mention!) have strong evidence for causing acute leukaemia in human via the general process of the AOP described here. Although direct observations on the initial in utero MIE in infant leukaemia are not possible, there is a lot of inferential evidence from animal and in vitro cellular studies suggesting strongly that infant leukaemia recapitulates at least at an apparent process level the treatment-related leukaemia. It is important to recognize that in therapy-related AML this has been clearly demonstrated with abnormalities affecting MLL locus. Chlorpyrifos is reported to be a Topo II poison and to induce MLL translocation in the human liver haematopoietic stem cells (Lu et al. 2015). However, it is probable that the dose dependence of the formation of DSBs and fusion genes is linear only in a very restricted “window” of dose range. Considering the rarity of IFL and the common exposure to Topo II poisons like bioflavonoids, specificity is

low. However, this consideration is limited by lack of experimental studies conducted with other than anticancer drugs on the sensitive target cells ie the liver haematopoietic stem cell.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Strong

DNA topoisomerases are key ubiquitous enzymes at all levels of living organisms. Important differences in sensitivity to topoisomerases inhibition might exist among different cell types and hematopoietic stem and progenitor cells can be a sensitive target during a critical developmental period. foetuses and newborns show that both the baseline and chemically induced micronuclei frequencies are higher in the foetus and infant than in adults.

The available evidence do not allow for evaluating whether any significant difference occurs among cell types or species in regard to the KE event " in utero MLL chromosomal translocation". Fetal liver hematopoietic stem cells are more susceptible to the tool chemical etoposide than maternal bone marrow mononuclear cells and this has been also observed in mouse.

The AO "infant lekaemia" is a pediatric leukaemia and in animals the disease is not known and the artificial reproduction of the disease in animal models have limitations.

Essentiality of the Key Events

In line with the defining question, essentiality for this AOP is moderate. However, the actual knowledge of the IFL is supporting the evidence that IFL is a “single hit” developmental disease and MLL translocation is an essential KE based on the probability linking MLL translocation and the occurrence of the disease. Based on this the overall essentiality can be considered moderate to strong.

Essentiality of the KEs; WoE analysis

		High (Strong)	Moderate	Low(Weak)
Support for Essentiality of KEs	**Defining Question** Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs (e.g. stop/reversibility studies, antagonism, knock out models, etc.)	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE leading to increase in KE down or AO	No or contradictory experimental evidence of the essentiality of any of the KEs
		Although there are no direct experimental studies to demonstrate that blocking action of TopoII poisons would prevent the AOP, there are considerable evidence for the relationship between the		

MIE In utero exposure to DNA topoisomerase II poison	MODERATE	concentration of etoposide and the formation of the MLL rearrangements in human (pre)haematopoietic progenitor/stem cells, which strongly suggest the essentiality of TopoII inhibition (e.g. Bueno et al 2009; Nanya et al 2015). In addition, chemical-induced DNA breakpoints are associated with predicted Topo II cleavage sites (ie MLL), supporting an essential role for TOPO II mediate breakage (Hernandez and Menendez 2016; Montecucco et al 2015). In human patients, therapy-related acute leukaemia characterized by MLL rearrangement is predominantly associated with etoposide treatment (Super et al. 1993)
KE1 In utero MLL chromosomal translocation	MODERATE.	Growing 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; Sanjuan-Pla et al 2015; Greaves 2015). Therefore, the totality of evidence suggests the ***essential*** role of the formation of MLL-partner fusion gene and product in causing pleiotropic effects in the affected cell and directing it to the obligatory pathway to the adverse outcome of leukaemia. The MLL-AF4 fusion gene is present in bone marrow mesenchymal stem cells in infant leukaemia patients, but not in patients of childhood leukaemia, suggesting that the origin of the fusion gene is probably prehaematopoietic and essential for development of IFL (Menendez et al 2009). TopoII ‘poisons’ etoposide and bioflavonoids (and some other chemicals) promote MLL rearrangements in in vitro prenatal cells or in utero. There are in vitro cellular and n vivo xenograph studies demonstrating that upon inhibiting signalling pathways from the fusion product on, cells can resume differentiation or clonal expansion of fusion gene-carrying cells is prevented (Benito et al 2015; Buechele et al 2015; Chen and Armstrong 2015). However, in absence of a relevant in vivo experimental model these findings are suggestive but not yet totally convincing.

Weight of Evidence Summary

Biological plausibility.

The biological plausibility for this AOP is strong. The relationship between DNA double strand breaks, MLL chromosomal translocation and infant leukaemia is well established. The same pathway is reproducible in chemotherapy-induced acute leukaemia in patients following treatment with etoposide, a known Topo II poison.

	Defining Question	**High (Strong)**	**Moderate**	**Low(Weak)**
1 Support for Biological Plausibility of KERs	Is there a mechanistic (i.e. structural or functional) relationship between KEup and KE down consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance	The KER is plausible based on analogy to accepted biological relationships, but scientific understanding is not completely established	There is empirical support for a statistical association between KEs but the structural or functional relationship between them is not understood
		Rationale: Although type II topoisomerases are essential to cell proliferation and survival, they have a significant genotoxic potential consequent to the resulting (double) strand breaks. Mis-repair of accumulated of DNA double strand breaks can result in chromosomal translocations which can persist in survived cells (Mc Clendon et al. 2009). Studies on identical twins and neonatal blood samples strongly implicate an		

MIE à KE1 In utero exposure to DNA topoisomerase II poison leads to In utero MLL chromosomal translocation	STRONG	in utero occurrence of the KER (Sanjuan-Pla et al 2015). Furthermore, a study in pregnant mice demonstrates that in utero exposure of the foetus to etoposide causes the MLL chromosomal translocation analogous to the human translocation except the principal fusion partner (Nanya et al 2015). Indirect evidence from human prehaematopoietic/mesenchymal stem cells and foetal liver haematopoietic progenitor and stem cells strengthen the plausibility. Experimental evidence in these cell lines has demonstrated that etoposide as a TopII poison causes DSBs in MLL and partner genes, which leads to the formation of fusion genes and their products (SanjuanPla et al 2015). MLL translocation sites (breakpoint sequences) in the therapy-related leukaemia fall within a few base pairs of etoposide-induced enzyme-mediated DNA cleavage site. Although rearrangements associated with infant leukaemias are often more complex than those observed in treatment-related leukaemias, many are nevertheless associated with stable TopII-mediated DNA cut sites (Cowell and Austin 2012; Pendleton et al 2014)
KE1 à AO In utero MLL chromosomal translocation leads to Infant leukaemia	STRONG	Rationale: The basic processes underlying overt leukaemia development are well understood and accepted. There is a general understanding of the molecular and epigenetic mechanisms leading to differentiation blockage and clonal expansion and there is evidence that the principal MLL-fusion genes and proteins harbour the necessary properties to execute the pathways associated with differentiation blockage and clonal expansion (Benito et al 2015; Chen and Armstrong 2015; Chen et al 2015).

Empirical support

The overall empirical support, using the chemical tool etoposide, is moderate. In vivo and, mainly in-vitro, experiments exist but they are lacking a clear dose or concentration response relationship.

	Defining Question	**High (Strong)**	**Moderate**	**Low(Weak)**
3 Empirical support for KERs	Does the empirical evidence support that a change in the KEup leads to an appropriate change in the KE down? Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup higher than that for KE down? Are inconsistencies in empirical support cross taxa, species and stressors that don't align with expected pattern of hypothesized AOP?	Multiple studies showing dependent change in both exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance) and no or few critical data gaps or conflicting data.	Demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor (ie endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesized AOP
MIE à KE1 In utero exposure to		Rationale: Evidence comes from in vitro studies in appropriate human cells and from an in vivo/ex vivo study in pregnant mice; the stressor has been		

DNA topoisomerase II poison leads to In utero MLL chromosomal translocation	MODERATE	etoposide in most of the experiments (Libura et al 2005; Whitmarsh et al 2003; Lovett et al 201, Nanya et al 2015). Some evidence to back this KER comes from in vitro studies with bioflavonoids, especially quercetin, genistein and kaempferol (Barjesteh et al 2007).
KE1 à KE2 In utero MLL chromosomal translocation leads to Infant leukaemia	MODERATE	Rationale: There are a number of factors and pathways linking the fusion products with differentiation blockage and clonal expansion (Marschalek 2010; Sanjuan-Pla et al 2015). *MLL* encodes a protein homologous to the Drosophila trithorax gene, which has relevant functions in embryogenesis and haematopoiesis (Ernest et al 2004, Hess et al 1997). Studies with *MLL-AF4*, *MLL-AF9* and *MLL-ENL* (Barabe et al 2007; Mulloy et al 2008) have clearly demonstrated how *MLL* chromosomal rearrangements block differentiation and enhance clonal expansion. However, there is a specific need to execute these studies in an appropriate experimental system with a proper target cell within a proper molecular and physiological environment. There are several animal models, in which *MLL*-rearranged fusion genes have been expressed and leukaemia developed (Chen et al 2006; Metzler et al 006; Krivtsov et al 2008; Bursen et al 2008; Tamai et al 2011). Engineered human hematopoietic stem and progenitor cell carrying an *MLL* rearrangement showed that a subset of cells persisted over time and demonstrated a higher clonogenic potential in colony forming assay (Breese et al. 2015). Cells engineered to carry *MLL-AF9* and *MLL-ENL* fusions demonstrated leukaemogenicity especially after ex vivo and repeated transplantation (Buechele et al 2015).

Uncertainties and Inconsistencies

- In utero evidence of the disease is difficult to obtain in humans and one has to resort to in vitro cellular systems, which may be inadequate to take into consideration the potential effects of proposed microenvironments, rapidly changing developmental stages and facilitating and modifying factors
- Animal models are a possibility (e.g. Nanya et al 2015), but are naturally prone to species-specific factors.
- An important problem is to provide a convincing and experimentally justified explanation for the dilemma between the rarity of disease in the face of pervasive exposure to topoII inhibitors
- The treatment-related AML apparently is a true surrogate for the infant leukaemia, at least mechanistically. Is it only because of etoposide as a principal chemical intiator has provided many crucial findings for understanding the infant leukaemia.
- The ‘poisoning’ of the TopoII-DNA cleavage complex has not been shown in the putative target cell, which is still not unequivocally identified.
- MLL-AF4 knock-in mice develop leukaemia only after a prolonged latency (e.g. Chen et al 2006), thus not recapitulating the ‘pathognomonic’ feature of infant leukaemia.
- The inability of available in vivo models to recapitulate the whole AOP process is due to a crucial factor which has not yet been found, or to model-specific peculiarities.
- In the face of the rarity of the disease, epidemiological studies especially concerning aetiology and risk factors are not powerful enough to provide robust answers. For instance, investigating the hypothesized relationship of bioflavonoids with infant leukaemia will have to consider the gap between the widespread intake of these phytochemicals and the very rare occurrence of the disease.
- The biology of the disease (i.e. IFL) and the experimental studies conducted with etoposide, indicate in-utero exposure of hematopoietic stem cells (HSC) as the most critical, if not essential, factor for the development of the A . However, a clear comparative quantification in terms of dose response vs different time of exposure

and cell systems is lacking.

- The very early embryonic structure and the liver haematopoietic stem cells in particular, are representing the target cell for this AOP. A clear understanding of a higher sensitivity of HSC vs, mature hematopoietic cells, particularly in the standard genotoxicity test battery is lacking and more chemicals and comparative assays should be tested to scientifically validate this cell system..
- What would be consequences if we say that the AOP is biologically possible, feasible, even probable, and then say that most of the evidence is impossible to get directly and has to be based on surrogates?

Quantitative Consideration

The WOE analysis indicates that many KEs and KERs lack especially experimental evidence, but overall the analysis supports the qualitative AOP. The strong element in the development of the qualitative AOP is the biological plausibility of the overall pathway that it can partially be based on studies in human treatment-related disease recapitulating many crucial features of the infant leukaemia. The lack of sufficient experimental data and uncertainties in quantitative information from treatment-related acute leukaemia makes it problematic to build convincing dose (concentration)-response and response-response relationships and to identify possible practical thresholds for stressors. The MIE is expected to show a dose response relationship to a certain extent. However, it is probable that the dose dependence of the formation of DSBs and fusion genes is linear only in a very restricted “window”. In too-low concentrations the outcome of the stressor is a successful repair of the break, in too-high concentrations the outcome is cell death. It should be kept in mind additionally that the quantification of dose-responses should also consider the different sensitivity of cell systems that should be also representative of the specific time-window of exposure (i.e. in-utero).

The most pressing future need is an adequate and robust experimental model system for the evaluation of relationships between doses, concentrations and responses within a temporal framework of the AOP.

Considerations for Potential Applications of the AOP (optional)

Applicability of the AOP

The proposed AOP is strictly life stage-dependent, being linked with in utero exposure and early embryogenesis. However, the surrogate disease (i.e. chemotherapy-related acute leukaemia) is not life stage restricted as well as the genotoxic hazard is not expected to be life stage related.

Potential regulatory applications of the AOP

This AOP was initiated with the intention to use an epidemiologically proposed human health outcome as AO and build back an AOP leading to this. Infant childhood leukaemia is a human disease and consequently apical regulatory endpoints can only explore the hazard by means of surrogate testing. These include carcinogenesis assays and blood cell analyses in the in vivo toxicology assessment. Considering the unique biology of this AO, these tests show some technical limitations and also the sensitivity and specificity of the available tests for the AO is limited. Additionally, experimental animal models replicating the AO are limited. Technical limitations of the standard regulatory tests include: Standard carcinogenesis studies do not include an early in-utero exposure time, blood cell analysis is not a standard requirement in the extended multi-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 these tests to capture this hazard is likely to represent a big hurdle and the regulatory tests are unlikely to represent the best way to explore this AO.

This AOP is however indicating that the MIE and the KE1 can be measured in scientific and/or regulatory validated test assays.

With these premises, the authors support the use of this AOP during the process of assessment of epidemiological studies and the use of the AOP framework to support the biological plausibility of the effects observed in the epidemiological studies when experimental and toxicological studies are indicative that the AOP is affected and this should guide on which additional studies should be performed, if the case, to integrate the AOP framework into the MOA framework for specific chemical entities.

In addition, this AOP should serve in guiding testing strategy. This include 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.liver HSPC).

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