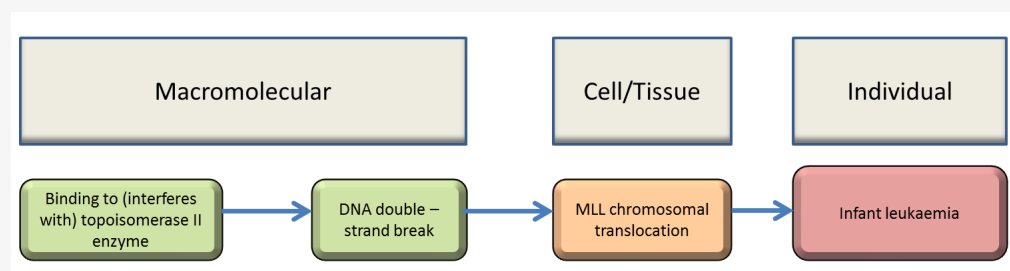


AOP ID and Title:

AOP 202: Inhibitor binding to topoisomerase II leading to infant leukaemia

Short Title: topoisomerase II binding, infant leukaemia

Graphical Representation



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Abstract

Infant leukaemia is a rare haematological disease (1 in 10⁶ 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 (including youngs and middle age adults) 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.

Following these distinct features a Molecular Initiating Event (MIE), two Key Event (KE) and an Adverse Outcome (AO) were identified. The MIE was identified as " DNA topoisomerase II poisons (interfers with) topo II enzyme" and epidemiological studies suggest that exposure to topoisomerases II poisons may be involved in generation of the two KEs, DNA double strand break and *MLL* chromosomal rearrangement.

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 driving 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 model 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. The authors recognize that additional elements are limiting the strenght of this AOP. Although a strong empirical support exists for the direct link between the MIE and the DNA double strand break and between this KE and the MLL translocation, the empirical support for the indirect link between MLL translocation and the AO is mainly based on one chemical stressor and that essentiality data are also limited and difficult to generate. The biological plausibility for the KERs is considered high for the initial step but is only moderate for the final step because of the uncertainties associated with lack of knowledge in the final step of the disease and lack of appropriate models able to fully recapitulate the disease. The empirical support for the KERs is overall considered moderate, as the relevant data only exist for etoposide and evidence is mainly indirect and based on the evidence from the therapy associated acute myeloid leukaemia. Therefore, the overall biological plausibility is considered moderate and the empirical support is also moderate

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 indicate an *in utero* origin at an early phase of foetal development. Investigation of identical twin pairs with infant leukaemia provided evidence of in-utero transfer of leukemic cells from one twin to the other (Ford AM, 1993), and the in-utero origin of this cancer was confirmed by retrospective analyses of neonatal blood spots from affected infants (Gale KB, 1997). The high concordance rate for leukaemia in monozygotic twins and the short latency of the disease suggest that MLL rearrangement in fetal hematopoietic stem cells causes infant leukaemia (Nanya M, 2015). 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 topo II inhibitor, Etoposide, induces DNA double-strand breaks between the S and the G2/M phases of the cell cycle and is related to the post treatment occurrence of the acutemyeloid leukaemia, which is showing a similar pattern of genetic changes as observed in the Infant Leukaemia (IFL) disease. Indeed, the hallmark of the IFL and acute myeloid leukaemia is the formation of *MLL* gene rearrangements (MLLr) via TopoII poisoning, leading to fusion genes and eventually acute leukaemia by global (epi)genetic dysregulation. Current knowledge supports the possibility that MLL-rearrangement in infant leukaemia is caused by transplacental exposure to topo2 poisons. Although it is considered unusual for a pregnant woman to be directly exposed to drugs such as etoposide, other compounds presents in the environment may exert similar effects, and this is considered toxicologically relevant for risk assessment (Nanya M, 2015). This 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. This 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, this 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. This AOP was first developed by the EFSA PPR Panel as part of a Scientific Opinion and published in the EFSA Journal 2017;15(3):4691 DOI: 10.2903/j.efsa.2017.4691. A copyright for figures and for most of the references included in this AOP was delt with in the EFSA Scientific Opinion. In addition, EFSA granted a research project for assessing *in vitro* and *in vivo* the potential genotoxic contribution of etoposide, Permethrin and Chlorpyrifos in human hematopoietic stem and progenitor cells (HSPCs) at different ontogeny stages, spanning from embryonic to adult HSPCs, with a special emphasis in their ability to induce *MLL* breaks/damage (Rodriguez et al. 2020)

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1252	Binding to (interferes with) topoisomerase II enzyme	Binding, topoisomerase II
2	KE	1461	DNA double-strand break	DSB
3	KE	1253	MLL chromosomal translocation	MLL translocation

Sequence	Type	Event ID	Title	IFL	Short name
4	AO	T254	Infant leukaemia		

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding to (interferes with) topoisomerase II enzyme	adjacent	DNA double-strand break	High	Not Specified
DNA double-strand break	adjacent	MLL chromosomal translocation	High	Not Specified
MLL chromosomal translocation	adjacent	Infant leukaemia	High	Not Specified

Stressors

Name	Evidence
Etoposide	High
Bioflavonoids	Low
Chlorpyrifos	Low
etoposide quinone	High

Etoposide

A number of drugs, environmental chemicals and natural substances are identified as Topoll “poisons” (Pendleton et al 2014) . 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 Topoll 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		
Epipodophyllotoxin	etoposide, teniposide	Montecucco et al 2015
Anthracyclines	doxorubicin, epirubicin, daunorubicin, idarubicin, aclarubicin	Cowell and Austin 2012
Anthracenedione	Mitoxantrone	Cowell and Austin 2012
Acridines	Amsacrine	Cowell and Austin 2012

Much of the relevant, albeit indirect, evidence to support this AOP come from the studies on etoposide, an anticancer drug Topoll “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.

Bioflavonoids

Bioflavonoids

Bioflavonoids are natural polyphenolic compounds in a large variety of plant-derived food items. Topoll-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).

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
		Ketron and Osheroff 2014
Isothiocyanates	benzyl-isothiocyanate, phenethyl-isothiocyanate, sulforaphane	
Other phytochemicals	Curcumin	Ketron and Osheroff 2014

Chlorpyrifos

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 as 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.

Environmental chemicals

Aromatic compounds	benzene, PAHs	
Nitrosamines	Diethylnitrosamine	Thys et al 2015
Organophosphates	Chlorpyrifos	Lu et al 2015

etoposide quinone

The properties of the quinone metabolite differed from those of etoposide, and the quinone appeared to function by a different mechanism. Previous studies with quinones and other protein-reactive agents have found that some of these compounds increase levels of topoisomerase II-mediated DNA cleavage by covalently adducting to the enzyme at residues that are distal to the active site. Thus, these agents are termed "covalent topoisomerase II poisons". It is believed that covalent poisons enhance DNA cleavage, at least in part, by closing the N-terminal gate of the protein. Several lines of evidence suggest that etoposide quinone poisons topoisomerase II α by this latter, covalent mechanism.

Overall Assessment of the AOP

Direct studies in humans are difficult or impossible to conduct 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 Topoisomerase II poison-caused acute leukaemia in children or adults) or from cellular and animal models. All cells have the two major forms of topoisomerases. Topoisomerases are able to alter the topological state of the DNA and topoisomerases are important targets for many chemotherapeutic agents and antibiotics (e.g. Fluoroquinolones). For examples, drugs that inhibit DNA topoisomerase II, such as doxorubicin and etoposide, can cause DNA damage. These agents prevent the DNA-resealing step which is normally catalyzed by topoisomerases. This AOP is mainly using etoposide for the KER empirical support as only for this agent an indirect link between MLL translocation and secondary leukaemia in cancer patients exist and the sequence of KEs (including the MIE) can be used to support an AOP specific for the AO i.e. Infant Leukaemia. Consequently, the biological plausibility for the KERs is considered high only for the initial step but is only moderate for the final step because of the uncertainties associated with lack of knowledge in the final step of the disease and lack of appropriate models able to fully recapitulate the disease. The empirical support for the KERs is overall considered moderate, as the relevant data only exist for etoposide and evidence are mainly indirect and based on the evidence from the therapy associated acute myeloid leukaemia.

1. Concordance of dose-response relationship

The only study in mice (Nanya et al 2016; conducted following in-utero exposure)) has shown that the dose of 0.5 mg/kg etoposide (day 13.5 of pregnancy) does not result in measurable etoposide concentration in foetal liver hematopoietic stem cells (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) and MLL translocation 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 myeloid 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 KE2. Available experimental models (Sanjuan-Pla et al 2015) are in conformation with this AOP, except that in the 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 Topo II 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 Topo II 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 model compound etoposide

	KE1	KE2	AO
Concentration of etoposide	DNA DSB consequent to topo II inhibition	MLL chromosomal rearrangement	Infant leukaemia
0.01 – 0.1 μ M, <i>in vitro</i> (TopII enzymes and cells in culture)	+++ (DNA damage response in various cells)	-	
0.1 – 1 μ M, <i>in vitro</i> cell cultures	+++ (haematopoietic progenitor and stem cells)	+	
0.5-5 μ M, <i>ex vivo</i> , mouse fetal liver HSC concentration ¹	+++ (inference from MLL cleavage)	+	- (no leukemia development)
max 5 μ M, <i>ex vivo</i> , mouse fetal liver HSC concentration ¹	+++ (inference from MLL cleavage)	+	- (no leukemia development)
Max >150 μ M, plasma concs in etoposide-treated patients ²	+++ (inference from MLL cleavage)	++ MLL-AF4 ³ fusion gene and protein	+

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

²plasma 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 MLL-AF4: Mixed lineage leukaemia gene with chromosomal translocation t(4;11)(q21;q23)

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

Chapter1.

Regarding the treatment-related acute myeloid leukaemia, strength, consistency and specificity of association of AO and MIE is strong, because only etoposide have strong evidence for causing acute leukaemia in human via the general process of the AOP described here. Evidence supporting the causal relationship between etoposide-induced TopoII inhibition, DSB and the MLL rearrangement leading to the fusion gene is also strong regarding treatment-related acute leukaemia. However, the evidence as such is indirect as it is occurring in an adult population and not following in-utero exposure and consequently; therefore, lacking a model able to reproduce the full IFL disease pattern, the overall biological plausibility is considered moderate and the empirical support is also moderate. The bioflavonoid-rich diet in pregnant women has been suggested to initiate infant leukaemia by an analogous causality between inhibition of TopoII enzymes in the target sensitive cells i.e. HSPC and creation of the fusion gene. However, there is no direct evidence in humans and it is also difficult or impossible to study. Power of epidemiological studies is relatively weak in the case of a very rare disease and case-control or spatio-temporal cluster studies have barely suggested a causal relationship between exposures and disease. Although the empirical support for the chemical stressor etoposide and the metabolite etoposide quinone should be considered strong, this still remains a limitation for the overall strenght of the weight of evidence for the empirical support. However, the biological plausibility linking topoII poisons to MLL rearrangements, when occurring in the appropriate cell population ie. prehematopoietic stem cell, is strong. Although direct observations on the initial MIE in infant leukaemia are not possible, there is a lot of inferential evidence from animal and in vitro cellular studies suggesting that infant

leukaemia recapitulates, at least at an apparent process level, the treatment-related adult leukaemia. It is important to recognize that in the 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. In addition, the effect described by Lu et al. 2015 (induction of MLL translocations through caspase 3-dependent genomic instability and Topoisomerase II inhibition in human fetal liver hematopoietic stem cells) was not reproduced by Rodriguez et al. 2020. Considering the rarity of IFL and the common exposure to Topo II poisons like bioflavonoids, specificity is therefore low. However, this consideration is limited by lack of experimental studies conducted with other than anticancer drugs on the sensitive target cells .e. the liver haematopoietic stem cell. Exposure to etoposide is directly linked to DSB, which is directly linked to MLL translocation, which is only indirectly linked to secondary myeloid leukemia in cancer patients; as the MIE and first two KEs have more empirical evidence to support their relationship. There is also empirical evidence that exposure to topo II inhibitors increases the incidence of MLL. The strengths of these relationships would not be diminished by the fact that what happens once the MLL is formed would be more complicated and less established if the MLL is occurring in the foetus.

Domain of Applicability

Life Stage Applicability

Life Stage Evidence

Embryo High

Taxonomic Applicability

Term Scientific Term Evidence Links

human Homo sapiens High [NCBI](#)

Sex Applicability

Sex Evidence

Unspecific High

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 " MLL chromosomal translocation". Fetal liver hematopoietic stem cells are more susceptible to the model chemical etoposide than maternal bone marrow mononuclear cells and this has been also observed in mouse.

The AO "infant leukaemia" 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

	Defining Question	High (Strong)	Moderate	Low(Weak)
Support for Essentiality of KEs	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
MIE Binding to (interfers with) topoisomerase II enzyme	MODERATE	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 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 DNA-DSB	STRONG	Topoisomerases are nuclear enzymes taht play essential role in DNA replication, transcription, chromosome segregation and recombination. All cells have the type I and type II enzymes. Etoposide, a Topo II inhibitor, kills cells by inhibiting the enzyme to ligate DNA (Smith 2014), which leads to the accumulation of DNA-DSBs. DNA-DSBs are indeed critical lesions resulting in a wide variety of genetic alterations including traslocations (Shirvastav 2008). Persistent or incorrectly		

		repaired DSBs can result in chromosome loss, deletion, translocation, or fusion, which can lead to carcinogenesis (Raynard 2017)
KE2 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 Infant 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 vitro prenatal cells or in utero. There are in vitro cellular and in 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. Many fusion proteins have been shown to recruit disruptor of telomeric silencing 1-like (DOT1L). Although DOT1L is not genetically altered in the disease <i>per se</i> its mislocated enzymatic activity is a direct consequence of the chromosomal translocation. The enzymatic activity of DOT1L is critical to the occurrence of MLL because methyltransferases-deficient DOT1L is capable of suppressing growth of MLL rearranged cells. A small-molecule inhibitor of DOT-1L inhibits cellular H3K79 methylation, blocks leukaemogenic gene expression, and selectively kills cultured cells bearing MLL-translocation (Chen and Armstrong 2015). Animal models expressing MLL-AF4 fusion gene exist (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 the models though latency is protracted (Sanjuan-Pla et al., 2015). Expression of the <i>MLL-AF4</i> (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. The <i>MLL-AF4</i> 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). Lin et al. (2016) designed a fusion gene between human MLL and murine af4 and demonstrated that it could transform—<i>via</i> retroviral transduction—human CD34⁺ cells to generate pro-B-ALL with all the characteristic features of the <i>MLL-AF4</i> infant leukaemia.

Weight of Evidence Summary

Biological plausibility.

The biological plausibility for this AOP is overall moderate. The relationship between Topo II inhibition, DNA double strand breaks, MLL chromosomal translocation and infant leukaemia is well established. Although this pathway is reproducible in chemotherapy-induced acute myeloid leukaemia in patients following treatment with etoposide, a known Topo II poison, direct evidence for IFL is not available

	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 relationship, 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:		

MIE to KE1 Binding to (interferes with) topoisomerase II enzymes, leads to DNA-DSB	STRONG	Although type II topoisomerases are essential to cell proliferation and survival, they have a significant genotoxic potential consequent to the resulting (double) strand breaks following enzymes inhibition. 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, Raynard 2017).
KE1 to KE2 DNA-DSB leads to MLL chromosomal translocation	STRONG	Rationale: Studies on identical twins and neonatal blood samples support the biological plausibility of this KER (Sanjuan-Pla et al 2015). Furthermore, a study in pregnant mice demonstrates that exposure of the foetus to etoposide causes DNA-DSB and MLL chromosomal translocation analogous to the human translocation except the principal fusion partner (Nanya et al 2015). 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)
KE2 to AO MLL chromosomal translocation leads to Infant leukaemia	MODERATE	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). However, due to the complexity of the AO, the biological plausible link can only be indirectly established using the cancer therapy acute leukaemia following treatment with etoposide as a model.

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. In addition, only one chemical, etoposide, is considered for the empirical support.

	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	Multiple studies showing dependent change in both exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance)	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	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

	empirical support cross taxa, species and stressors that don't align with expected pattern of hypothesized AOP?	and no or few critical data gaps or conflicting data.	considerations, differences among laboratories, etc.	don't align with expected pattern for hypothesized AOP
MIE to KE 1 Binding to (interferes with) topoisomerase II leads to DNA DSB.	STRONG	Rationale: Experimental evidence in pre-hematopoietic/mesenchymal 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). In standard eukariotic cell models, production of DNA DSB is the expected outcome following treatment with Topo II poisons chemotherapeutic or antibiotic agents.		
KE1 to KE2 DNA-DSB leads to MLL chromosomal translocation	MODERATE	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 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).		
KE2 to AO 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). <i>MLL</i> encodes a protein homologous to the <i>Drosophila trithorax</i> gene, which has relevant functions in embryogenesis and haematopoiesis (Ernest et al 2004, Hess et al 1997). Studies with <i>MLL-AF4</i>, <i>MLL-AF9</i> (t(9;11)(p22;q23)) and <i>MLL-ENL</i> (t(11;19)(q23;p13.3)) (Barabe et al 2007; Mulloy et al 2008) have clearly demonstrated how <i>MLL</i> chromosomal rearrangements block differentiation and enhance clonal expansion There are several animal models, in which <i>MLL</i>-rearranged fusion genes have been expressed and leukaemia developed (Chen et al 2006; Metzler et al 2006; Krivtsov et al 2008; Bursen et al 2008; Tamai et al 2011). Engineered human hematopoietic stem and progenitor cell carrying an <i>MLL</i> 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 <i>MLL-AF9</i> and <i>MLL-ENL</i> fusions demonstrated leukaemogenicity especially after ex vivo and repeated transplantation (Buechele et al 2015).		

Uncertainties and Inconsistencies

- Evidence of the MIE 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 microenvironments, rapidly changing developmental stages and facilitating and modifying factors like the one occurring in early phase of development
- 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.
- 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.

- 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 initiator 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 AO. 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 (e.g. chromosome aberration, micronucleus test, mouse lymphoma test) is lacking and more chemicals and comparative assays should be tested to scientifically validate this cell system.
- The role of fusion partners in the process of leukaemogenesis has not been completely elucidated and is representing an important uncertainty for this AOP. Normally, all of them participate in chromatin modifying complex, for example, acting on the transcriptional regulation of target genes. The MLL fusion proteins are dysregulating this highly regulated process and probably different fusion partners are working in a distinct way with variable modulatory effect on signalling pathways in leukaemic cells. Recruitment of DOT1L or officially KMT4, a histone methyltransferase, seems to be a common feature of many oncogenic MLL fusion proteins, resulting in the over methylation and overexpression of several MLL target genes encoding for transcription factors involved in body patterning and hematopoiesis. It is indeed possible that an additional (epi)genetic KE would occur downstream to MLLtranslocation, but a better understanding of the role of fusion partners in the process of leukaemogenesis would be necessary before adding it and at the moment this should be considered as a knowledge gap for this AOP.
- 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.
- 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.

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, able to recapitulate the KEs included in this AOP, 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 AO 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 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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Appendix 1

List of MIEs in this AOP

Event: 1252: Binding to (interferes with) topoisomerase II enzyme

Short Name: Binding, topoisomerase II

Key Event Component

Process	Object	Action
DNA topoisomerase II activity		abnormal

AOPs Including This Key Event

AOP ID and Name

Event Type

[Aop:202 - Inhibitor binding to topoisomerase II leading to infant leukaemia](#) MolecularInitiatingEvent

Stressors

Name

Etoposide

etoposide quinone

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Etoposide is one of the most well studied topoisomerase II targeted agents. It stabilizes covalent topoisomerase II-cleaved DNA complexes (ie cleavage complexes) by interacting at the enzyme-DNA interface in a noncovalent manner (Smith et al. 2014).

Etoposide (and its derivatives) stimulate DNA cleavage mediated by yeast topoisomerase II. As a result of etoposide action, high levels of topoisomerase II-associated DNA breaks accumulate in treated cells (Hande et al. 1998; Ross et al 1984; Wistelrman et al.2007).

Etoposide quinone induces DNA cleavage via an enzyme-mediated mechanism. Control reactions were conducted in the absence of enzyme or drug (DNA Control), in the presence of 30 μ M etoposide quinone without enzyme (+EQ -hTII β), or in the presence of topoisomerase II β without drug (-EQ +hTII β). The quinone induced $\sim$ 4 times more enzyme-mediated DNA cleavage than did the parent drug. Furthermore, the potency of etoposide quinone was $\sim$ 2 times greater against topoisomerase II β than it was against topoisomerase II α , and the drug reacted $\sim$ 2–4 times faster with the β isoform. Etoposide quinone induced a higher ratio of double- to single-stranded breaks than etoposide, and its activity was less dependent on ATP (Smith et al. 2014).

TOP2 isoforms covalently bound to genomic DNA in hESC after 15 min treatment with etoposide 1-100uM (Rodriguez et al.2020).

Etoposide

Etoposide is one of the most well studied topoisomerase II-targeted agents in clinical use. The drug stabilizes covalent topoisomerase II-cleaved DNA complexes (i.e., cleavage complexes) by interacting at the enzyme–DNA interface in a noncovalent manner. Once the double helix is cut, the drug slips (i.e., intercalates) between the 3′-hydroxyl and the enzyme-linked 5′-phosphate at the cleaved scissile bond and acts as a physical block to topoisomerase II-mediated DNA ligation. Etoposide and other drugs that utilize this mechanism are termed “interfacial topoisomerase II poisons”. The catechol displayed properties that were similar to those of the parent drug and appeared to be an interfacial poison. The properties of the quinone metabolite differed from those of etoposide, and the quinone appeared to function by a different mechanism. Previous studies with quinones and other protein-reactive agents have found that some of these compounds increase levels of topoisomerase II-mediated DNA cleavage by covalently adducting to the enzyme at residues that are distal to the active site. Thus, these agents are termed “covalent topoisomerase II poisons”. It is believed that covalent poisons enhance DNA cleavage, at least in part, by closing the N-terminal gate of the protein. Several lines of evidence suggest that etoposide quinone poisons topoisomerase II by this latter, covalent mechanism (Smith NA, 2014).

etoposide quinone

Etoposide metabolites, ie etoposide quinone, is also a potent topoisomerase II β poisons. The quinone is able to induce about 4 times more enzyme-mediated DNA cleavage than does the parent drug. Furthermore, the potency of etoposide quinone was about 2 times greater against topoisomerase II β than it is against topoisomerase IIP, and it reacts about 2 to 4 times faster with the β isoform. The quinone metabolite induces a higher ratio of double - to single strand breaks than the parent chemical, and its activity is less dependent on ATP. Whereas etoposide acts as an interfacial topoisomerase II poison, etoposide quinone displayed all of the hallmarks of a covalent poison: the activity of the metabolite was abolished by reducing agents, and the compound inactivated topoisomerase II β when it was incubated with the enzyme prior to the addition of DNA (Smith et al. 2014).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

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 fetuses and newborns show that both the baseline and chemically induced micronuclei frequencies are higher in the fetuses and infants than in adults (Udroui 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 (Udroui et al. 2016). The existence of relevant interspecies differences is unknown, but it cannot be ruled out presently.

Key Event Description

Type II topoisomerases are ubiquitous enzymes that are required for proper chromosome structure and segregation and play important roles in DNA replication, transcription, and recombination. Type II topoisomerases change DNA topology by breaking and rejoining double-stranded DNA. These enzymes can introduce or remove supercoils and can separate two DNA duplexes that are intertwined. Type II topoisomerases relax DNA and remove knots and tangles from the genetic material by passing an intact double helix (transport segment) through a transient double-stranded break that they generate in a separate DNA segment (gate segment). Humans encode two closely related isoforms of the type II enzyme. Topoisomerase II P is essential for the survival of proliferating cells and topoisomerase II β plays critical roles during development. However, because these enzymes generate double-stranded DNA breaks during their crucial catalytic functions, the consequences are not only beneficial. Although essential to cell survival, they also pose an intrinsic threat to genomic integrity every time they act. Beyond their critical physiological functions, topoisomerase IIP and II β are the primary targets for some of the most active and widely prescribed drugs currently used for the treatment of human cancers. These agents kill cells by increasing levels of covalent topoisomerase II-cleaved DNA complexes that are normal, but fleeting, intermediates in the catalytic DNA strand passage reaction. Many chemicals do so by inhibiting the ability of the type II enzymes to ligate cleaved DNAs. When the resulting enzyme-associated DNA breaks are present in sufficient concentrations, they can trigger cell death pathways. Chemicals that target type II enzymes are referred to as topoisomerase II poisons because they convert these indispensable enzymes to potent physiological toxins that generate DNA damage in treated cells. 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).

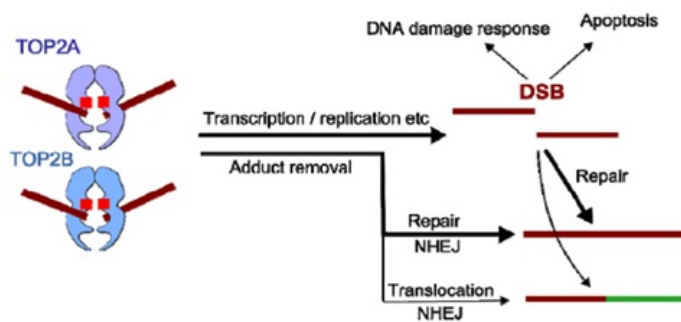


Fig.33: TOP2 Poisons, downstream events. TOP2 poisons inhibit the religation step of the TOP2 reaction cycle, leading to accumulation of covalent TOP2-DNA cleavage complexes. These lesions are cytotoxic and lead to activation of the DNA damage response and potentially apoptosis. Alternatively these lesions 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)

DNA topoisomerase (Topo) 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 al 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.

The MIE, topo II poisons, can occur prenatally i.e. prenatal exposure to topo II poisons. Human embryonic stem cells are more sensitive to topo II inhibition than postnatal CD34+ cells, linking embryonic exposure to topoisomerase II poisons to genomic instability. However, little is known about the nature of the target cell for transformation (Bueno et al. 2011).

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 which bind selectively to interfaces as macromolecular machines assemble which bind selectively to interfaces as macromolecular machines assemble. 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.

Topoisomerase poisons stabilize the covalent enzyme–DNA complex. There are several key characteristics of this complex: it includes protein covalently bound to DNA as well as a strand break in the DNA substrate, and it is also freely reversible. Accordingly, if the chemical is removed the enzyme rapidly reseals the DNA. Covalent complexes are quantified in two ways: by measuring the levels of protein covalently bound to DNA or by directly assaying for DNA strand breaks in the presence of topoisomerase and test agent or known drug. The assay directly measures DNA strand breaks induced by topoisomerase I in a substrate that carries a strong DNA cleavage site. Similarly, the plasmid linearization assay measures double strand breaks induced in plasmid DNA by topoisomerase II. The Alternate Protocol allows for the visualization of breaks induced on a larger substrate. Different protocols are used to measure the amount of the cleavage complex by determining the levels of topoisomerases that are covalently associated with DNA. Since the covalent complex is a normal step in the topoisomerase reaction, it can be detected (using very sensitive assays) even in the absence of a topoisomerase poison. However, addition of a topoisomerase poison greatly increases the levels of covalent complexes. Protocol and procedure details for measuring topoisomerase inhibition are fully reported in Nitiss et al. 2012.

In vivo complex enzyme assay (Rodriguez et al. 2020): hESCs were either immediately lysed in 1 % (w/v) sarkosyl (Sigma L7414). Lysates were processed according to the in vivo complex of enzyme (ICE) assay (Nitiss, Soans, Rogojina, Seth, & Mishina, 2012;

Schellenberg et al., 2017). Briefly, sheared samples were centrifuged with a CsCl (Applichem-Panreac, A1098) gradient at 57,000 r.p.m. for 20 h at 25 °C using 3.3 ml 13 x 33 mm polyallomer Optiseal tubes (Beckman Coulter) in a TLN100 rotor (Beckman Coulter). For slot blotting, ICE samples containing 1, 2 or 4 µg of DNA were transferred onto Odyssey Nitrocellulose Membranes (LI-COR Biosciences) using a Bio-Dot SF Microfiltration Apparatus (Biorad). For western blot of ICE, samples were resuspended in 12,000 units of Micrococcal Nuclease (MNase, NEB 0247S), 1 x MNase buffer (NEB, B0247S) and 100 µg / mL BSA (NEB, B9000S), then incubated at 37 °C for 6 h. Samples were run in 10% SDS-PAGE and transferred to Immobilon-FL Transfer Membranes (Millipore). Membranes were blocked for 1 h in Odyssey Blocking Buffer (LI-COR Biosciences), then incubated for 2 h with primary antibodies in the same buffer with additional 0.1% (v/v) TWEEN 20, washed 3x with TBS-0.1%-TWEEN20, incubated with secondary antibodies for 1 h, and finally washed again. Once the membranes were dry, slots were analyzed and quantified in Odyssey CLx using ImageStudio Odyssey CLx Software.

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List of Key Events in the AOP

Event: 1461: DNA double-strand break

Short Name: DSB

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:202 - Inhibitor binding to topoisomerase II leading to infant leukaemia	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

DSB occurs in eukaryotic and procaryotic cells. There is good evidence for conservatism of DSB processing pathways in human cells (Gravel et al. 2008).

Key Event Description

DNA double-strand breaks (DSB) is formed as a consequence of the production of excision repair breaks opposite each other on the two strands of DNA, and by the production of an excision repair break opposite a DNA daughter-strand gap. DSB are considered to be critical primary lesions in the formation of chromosomal aberrations.

To repair this potentially lethal damage, eukaryotic cells have evolved a variety of repair pathways related to homologous and illegitimate recombination, also called non-homologous DNA end joining (NHEJ), which may induce small scale mutations and chromosomal aberration (Pfeiffer et al. 2000). Repair by NHEJ often leads to small deletions at the site of the DSB and is considered error prone. The second repair mechanism, the Homologous Recombination (HR) is directed by extensive homology in a partner DNA molecule. In mitotic cells NHEJ occurs throughout all phases of the cell cycle, whereas HR is largely restricted to the S and G2 phases when the sister chromatid is available to mediate the repair process (Reynard et al. 2017). Persistent or

incorrectly repaired DSBs can result in chromosome loss, deletion, translocation, or fusion, which can lead to carcinogenesis through activation of oncogenes or inactivation of tumor-suppressor genes (Raynard et al.2017). The DSB repair pathways appear to compete for DSBs, but the balance between them differs widely among species, between different cell types of a single species, and during different cell cycle phases of a single cell type. (Shrivastav et al. 2008).

DSBs are induced by agents such as ionizing radiation and chemicals that directly or indirectly damage DNA and are commonly used in cancer therapy (Shrivastav et al. 2008). DSBs also arise during DNA replication when the DNA-polymerase ensemble encounters obstacles such as DNA lesions or unusual DNA structures (Raynard et al 2017). Additional endogenous sources include reactive oxygen species, generated during cellular metabolism, collapsed replication forks and nucleases(Shrivastav et al. 2008) .

How it is Measured or Detected

A very early step in the cellular response to DSBs is the phosphorylation of a histone H2A variant, H2AX, at the sites of DNA damage. H2AX is rapidly phosphorylated (within seconds) at serine 139 when DSBs are introduced into mammalian cells resulting in discrete γ -H2AX (phosphorylated H2AX) foci at the DNA damage sites. H2AX phosphorylation also appears to be a general cellular response to processes involving DSB intermediates including V(D)J recombination in lymphoid cells and meiotic recombination in mice. Phosphorylation of H2A at serine 139 causes chromatin decondensation and appears to play a critical role in the recruitment of repair or damage-signaling factors to the sites of DNA damage. DNA DSB staining based on the phosphorylation of the histone H2A.X at serine 139 in response to DNA damaging agents which cause double strand breaks in cells that are cultured in microtiter plates is a rapid method for the identification and quantification of the damage (Seluanov et al.2002).

Microscopic examination of individual mammalian cells embedded in agarose, subjected to electrophoresis, and stained with a DNA-binding dye provides a way of measuring DNA damage and of assessing heterogeneity in DNA damage within a mixed cell population. (Olive P. et al. 1991).

Pulsed field gel electrophoresis (PFGE) is the main method used for measurement of DNA DSB in mammalian cells (Blocker D et al. 1989 and 1990, Stamato T et al 1990, Ager D et al 1990). Alternatively the DNA is size fractionated in the pulsed-field gel, and the weight fraction of DNA below a certain defined size is measured (Erixo K. et al. 1990, Stenelov B. et al. 1995). An additional method to measure prompt DSBs without including heat-labile sites is also reported (Stenelov B. et al. 2003).

In vitro assays for topoisomerase II based on the decantation of double strand DNA are extensively reported in Nitiss et al. 2012.

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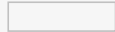
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Event: 1253: MLL chromosomal translocation

Short Name: MLL translocation

Key Event Component

Process	Object	Action
Translocation, Genetic		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:202 - Inhibitor binding to topoisomerase II leading to infant leukaemia	KeyEvent

Stressors

Name
Etoposide

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
embryonic cell

Evidence for Perturbation by Stressor

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). 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).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

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).

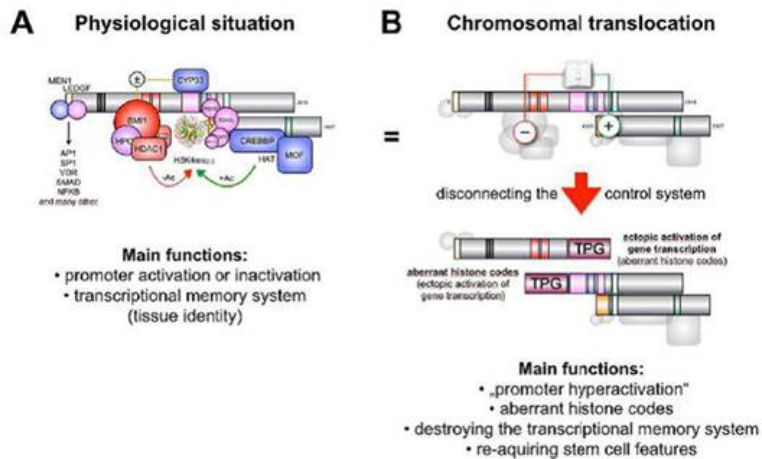
Key Event Description

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).

MLL, a human homologue of the epigenetic transcriptional regulator Trithorax of *Drosophila*, is an upstream transcriptional effector of *HOX* genes. The importance of normal MLL protein for normal axial-skeletal developmental process and *HOX* gene regulation has been demonstrated in the embryos of heterozygous and homozygous *MLL* knockout and *MLL* truncation mutant mice. Furthermore, expression of MLL protein is not necessary for turning on transcription of certain *HOX* genes, but for the maintenance of their transcription. Experiments *in vitro* using hematopoietic progenitors from embryos of homozygous *MLL* knockout mice or mice with *MLL* mutant showed that MLL was also critical for hematopoietic development. Recent findings suggested that MLL is required during embryogenesis for the specification or expansion of hematopoietic stem cells. As *HOX* genes also play a key role in the regulation of hematopoietic development, the hematopoietic dysfunction of *MLL* null cells is likely to be attributed to deregulated patterns of *HOX* gene expression in hematopoietic stem cells or progenitors. This link between MLL, *HOX* gene regulation, and hematopoiesis is of particular importance (Li et al. 2005).

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).

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).

How it is Measured or Detected

MLL rearrangements can be identified following different methods. It is worth noting that different methods will give a different information detail.

- **Split-signal FISH:** The split-signal FISH approach uses two differentially labeled probes, located in one gene at opposite sites of the breakpoint region. Probe sets were developed for the genes *TCF3 (E2A)* at 19p13, *MLL* at 11q23, *ETV6* at 12p13, *BCR* at 22q11, *SIL-TAL1* at 1q32 and *TLX3 (HOX11L2)* at 5q35. In normal karyotypes, two colocalized green/red signals are visible, but a translocation results in a split of one of the colocalized signals. Split-signal FISH has three main advantages over the classical fusion-signal FISH approach, which uses two labeled probes located in two genes. First, the detection of a chromosome aberration is independent of the involved partner gene. Second, split-signal FISH allows the identification of the partner gene or chromosome region if metaphase spreads are present, and finally it reduces false-positivity (Van der Burg et al, 2004).
- **RT-PCR in combination with long-distance inverse PCR (LDI-PCR)** performed on isolated genomic DNA. This method allows the identification of any kind of *MLL* rearrangement if located within the breakpoint cluster region. The method uses LDI-PCR to identify *MLL* translocations independent of the involved partner gene or other *MLL* aberrations that occurred within the *MLL* breakpoint cluster region. This method allows high-throughput analyses because genomic *MLL* fusion sequences can be obtained with a minimum of only four PCR reactions. Moreover, this method requires only small quantities of genomic patient DNA (1 µg) and provides relevant genetic information that can be used directly for quantitative minimal residual disease (MRD) analyses (Meyer et al. 2005).

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. FISH staining is however necessary for identification of MLL translocations.

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List of Adverse Outcomes in this AOP

[Event: 1254: Infant leukaemia](#)

Short Name: IFL

AOPs Including This Key Event

AOP ID and Name

Event Type

[Aop:202 - Inhibitor binding to topoisomerase II leading to infant leukaemia](#) AdverseOutcome

Stressors

Name

Etoposide

Biological Context

Level of Biological Organization

Individual

Evidence for Perturbation by Stressor

Etoposide

Topo II is a well validated anti-cancer target and Topo II poisons are widely used and effective therapeutic agents; but they are associated with the occurrence of late complications, including therapy-related acute leukaemia (Cowell and Austin, 2012). 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). *MLL* rearrangement, 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.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	High

Sex Applicability

Sex	Evidence
Mixed	High

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 (Hernandez 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.

Key Event Description

B cell Acute Lymphocytic Leukaemia (ALL) is the most frequent cancer in children. Infant leukaemia is a rare haematological disease with an incidence of 1 in 10⁶ 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:

- 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. Whereas about 60–80% of infant ALL carry an *MLL* (Sam et al., 2012; Jansen et al., 2007), for infant acute myeloid leukaemia (AML) the percentage of *MLL* is lower than 40%;
- 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. 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.

Clinically, IFL is characterised by 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 TG 443) include endpoints that can potentially explore the AO; however, considerations should be made on the specificity of the disease to humans. Indeed, IFL, as such, is not an animal disease and never reported as chemically induced outcome in cancerogenesis studies. Regarding the cancerogenesis studies, it should however be noted that they are generally performed in young adult animals and protocol including the treatment of the dams from the mating period are not common. For this reason, the sensitivity of the cancerogenesis study to capture this hazard is at its best unknown.

Regulatory Significance of the AO

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 studies 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. It is however worth noting that IFL is not an animal disease and therefore the outcome of a chemically induced MLL translocation can likely only be tested at KE levels. In addition, MLL translocation is clearly a common node to alternative AOs not described in this AOP (chemotherapy induced leukaemia) and genotoxicity per se should be considered as adverse.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

[Relationship: 1634: Binding, topoisomerase II leads to DSB](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibitor binding to topoisomerase II leading to infant leukaemia	adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Life Stage Applicability

Life Stage Evidence

Embryo High

Sex Applicability

Sex Evidence

Mixed Not Specified

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 fetuses and newborns show that both the baseline and chemically induced micronuclei frequencies are higher in the fetuses and infants than in adults (Udroui 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 (Udroui et al. 2016). The existence of relevant interspecies differences is unknown, but it cannot be ruled out presently.

Key Event Relationship Description

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). Indeed, 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). Topoisomerase poisons stabilize the normally transient topoisomerase-induced DSBs and are potent and widely used anticancer drugs (Cowell and Austin 2012). They interfere with the religation step in the topoisomerase II reaction cycle, leading to the accumulation of DNA DSBs. The inhibition of the religation step will result in the formation of an unusual type of DSB called a cleavage complex, in which the topoisomerase protein remains covalently coupled to the DNA (Cowell and Austin 2012).

Evidence Supporting this KER

All cells have two major forms of topoisomerases; Type I, which make single-stranded cuts in DNA, and Type II enzymes, which cut and pass double-stranded DNA (Nitiss et al 2012). Evidence supporting the causal relationship between etoposide-induced TopoII inhibition, DNA DSB and the MLL rearrangement leading to the fusion gene is strong regarding treatment-related acute leukaemia (Cowell and Austin 2012; Pendleton et al 2014).

Biological Plausibility

The KER as such is biologically plausible and strong. 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.

Empirical Evidence

A type II topoisomerase can introduce negative supercoils into DNA, all known eukaryotic cells can only relax DNA. The decatenation of interlocked DNA is a critical topoisomerase function, since semi-conservative DNA replication results in catenated sister chromatids. Topoisomerases are important targets for many chemotherapeutic agents. These agents convert their target topoisomerases to DNA-damaging agents. The DNA is cut in both strands and the agents prevent the subsequent DNA-resealing step normally catalyzed by topoisomerases (Nitiss 2009).

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).

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Relationship: 1635: DSB leads to MLL translocation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibitor binding to topoisomerase II leading to infant leukaemia	adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Sex Applicability

Sex Evidence

Mixed High

Seminal studies demonstrated an *in utero*, pre-natal origin of these *MLLr* in monozygotic twins with concordant B-ALL, in retrospective analysis in Guthrie cards and cord bloods (Ford et al., 1993; Gale et al., 1997). A single-pulse of etoposide induces DSBs measured by γ -H2AX staining in all primary cell types tested (hESC, fetal-, neonatal- and adult-derived CD34+ HSPCs) (Rodriguez et al. 2020).

Key Event Relationship Description

There is evidence that the inappropriate joining of 'hanging ends' following DSB 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).

Evidence Supporting this KER

Biological Plausibility

The KER as such is biologically plausible and strong. DNA strand breaks, 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; Money Penny et al 2006; Bueno et al 2009; Menendez et al 2009), which shows that DSB (in this specific case due to TopoII poisoning) -associated MLL rearrangements are produced in appropriate human cells in utero.

Empirical Evidence

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. A clear activation of DNA damage response was observed at the dose of 10 mg/kg (Nanya et al. 2016).

There is information about etoposide-induced DSB and MLL chromosomal translocation in human HSPCs at different ontogeny stages, spanning from embryonic to adult HSPCs.

In vitro, a single-pulse of etoposide induced DSBs measured by γ-H2AX staining in all primary cell types tested (hESC-, fetal-, neonatal- and adult-derived CD34⁺ HSPCs). In embryonic (hESC-derived) and neonatal (CB-derived) CD34⁺ HSPCs ETO showed a significant trend towards higher frequency of MLL breaks than control. Median fluorescence intensity (MFI) of γ-H2AX determined at 3h, 6h, and 12h after a single-pulse (0 h) exposure to 10µM etoposide rapidly (3 h) induced the expression of γ-H2AX regardless the cell type targeted. Fetal Bone Marrow, Cord Blood and Peripheral Blood -derived CD34⁺ HSPCs managed to partially repair the DSBs overtime (6 h and 12 h). In contrast, etoposide-induced DSBs seem irreversible in hESC (Rodriguez et al. 2020). MLL gene breaks was demonstrated upon single-pulse exposure to etoposide at 1µM in undifferentiated hESC and hESC-derived CD34⁺ hematopoietic cells (Rodriguez et al. 2020)

Uncertainties and Inconsistencies

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.

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[Relationship: 1331: MLL translocation leads to IFL](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibitor binding to topoisomerase II leading to infant leukaemia	adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	High

Sex Applicability

Sex	Evidence
Mixed	High

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. 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). These unique characteristics indicate that biological domain for this KER is specific to an early in-utero exposure condition.

Key Event Relationship Description

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).

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 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 TopoII-mediated DNA cut sites. Although all these findings are indirect regarding infant leukaemia, they are nevertheless rather persuasive in this respect.

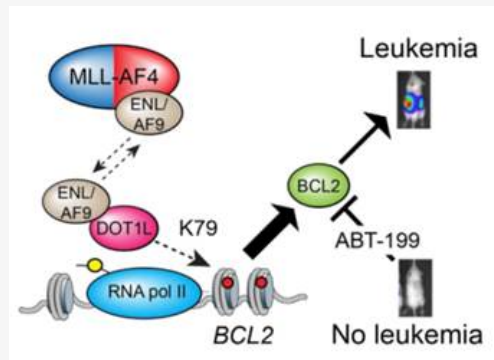
Growing scientific evidence, including the stable genome of the patients, suggests that infant leukaemia originates from one “big-hit” occurring during a critical developmental window of stem cell vulnerability (Andersson et al 2013; Greaves 2015). Therefore, the totality of evidence suggests the **essential** 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.

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 (t(10;11)(p12;q23) translocation, which encode the MLL AF10 fusion oncoproteins, observed in patients with acute myeloid leukemia), 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 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 hematopoiesis (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 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 lymphoblastic leukemia 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; Prele 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.

Evidence Supporting this KER

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 stem cell vulnerability (Andersson et al 2013; Greaves 2015). Different from the “two-hit” model of the adult leukemias, the infant leukemia is a developmental disorder where the clonal expansion is a direct consequence of in utero *MLL* translocation.

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.

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 Evidence

Secondary acute leukaemia carrying *MLL-r* is an adverse effect observed in patients treated with etoposide. Characteristics of the disease are in many ways analogous to those in infant leukaemia (Joannides et al., 2010, 2011). 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; Ezoe, 2012; Pendleton et al., 2014). It is recognised that the *MLL-r* fusion genes are caused by etoposide 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.

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. Etoposide 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" in animal model of human bone marrow cell graft graft (Rodriguez et al. 2020).

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 and 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.

The product of MLL and MLL-r fusion genes are histone methyltransferases (HMT), which are the best known epigenetic mechanisms involved in MLL-r infant ALL. The main epigenetic mechanism is that MLL is a HMT with a H3K4me3 genome-wide

profile. When MLL breaks and fuses to a partner it recruits DOT1L, the sole HMT H3K79me3 that thus regulates gene expression of the main MLL targets, namely HOX genes, MEIS, etc. Further epigenetic mechanisms recently proposed involved BCL2 activation through H3K70 methylation and H3K27 acetylation. Overall, the gene fusion products disrupt epigenetic pathways. The MLL-AF4 fusion protein binds to gene targets and is proposed to cause inappropriate gene activation through multiple transcription elongation and epigenetic mechanisms but further investigation is necessary to understand the exact connections between these factors and the enhancer activity.

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