AOP ID and Title:

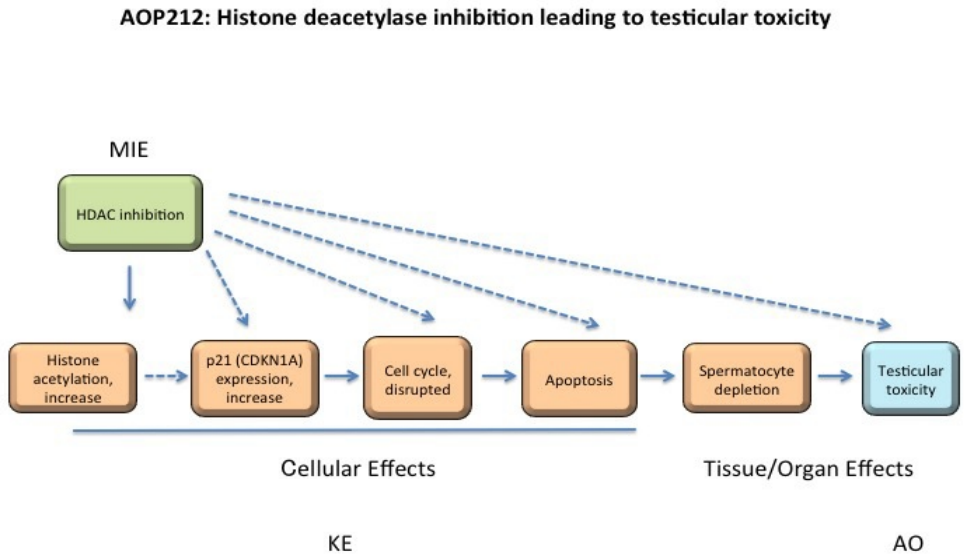
SNAPSHOT

Created at: 2019-11-08 12:37

AOP 212: Histone deacetylase inhibition leading to testicular toxicity

Short Title: Histone deacetylase inhibition leading to testicular toxicity

Graphical Representation



Authors

Shihori Tanabe, Akihiko Hirose, Takashi Yamada

Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	EAGMST Under Review	1.52	Included in OECD Work Plan

Abstract

Testicular toxicity is of interest for human health risk assessment especially in terms of reproductive and developmental toxicity, however, the testicular toxicity has not fully elucidated. HDIs are approved as anti-cancer drugs since HDIs have apoptotic effect in cancer cells. HDIs includes the short chain fatty acids (e.g., butyrate, valproate ,MAA), hydroxamic acids (e.g., SAHA, TSA), cyclic tetrapeptides (e.g., FK-228), benzamides (e.g., N-acetyldinaline and MS-275) and epoxides (depeudecin, trapoxin A), of which MAA especially focused on have the testicular toxicity such as testis atrophy *in vivo*. The intracellular mechanisms of induction of the spermatocyte apoptosis by HDIs are suggested as HDAC inhibition as MIE, histone acetylation increase, p21 expression increase, disrupted cell cycle, apoptosis, and spermatocyte depletion as KEs. Adverse outcome includes testicular toxicity. The HDIs inhibit deacetylation of the histone, leading to the increase in histone acetylation, followed by increase in p21 gene expression. The apoptosis induced by disrupted cell cycle leads to spermatocyte depletion and testis atrophy. We propose new AOP for HDAC inhibition leading to testicular toxicity. This AOP may be one of the pathways induced by HDIs, which suggests the networks of the pathways with hyperacetylations of cellular proteins other than histones.

Abbreviation: HDAC: histone deacetylase, HDI: HDAC inhibitor, SAHA: syberooylanilide hydroxamic acid, TSA: trichostatin A, MAA:

methoxyacetic acid, MIE: molecular initiating event, KE: key event, AOP: adverse outcome pathway

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1502	Histone deacetylase inhibition (https://aopwiki.org/events/1502)	Histone deacetylase inhibition
2	KE	1503	Histone acetylation, increase (https://aopwiki.org/events/1503)	Histone acetylation, increase
3	KE	1504	p21 (CDKN1A) expression, increase (https://aopwiki.org/events/1504)	p21 (CDKN1A) expression, increase
4	KE	1505	Cell cycle, disrupted (https://aopwiki.org/events/1505)	Cell cycle, disrupted
5	KE	1262	Apoptosis (https://aopwiki.org/events/1262)	Apoptosis
6	KE	1515	spermatocyte depletion (https://aopwiki.org/events/1515)	spermatocyte depletion
7	AO	1506	testicular toxicity (https://aopwiki.org/events/1506)	testicular toxicity

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Histone deacetylase inhibition (https://aopwiki.org/relationships/1709)	adjacent	Histone acetylation, increase	High	Moderate
Histone acetylation, increase (https://aopwiki.org/relationships/1710)	adjacent	p21 (CDKN1A) expression, increase	Moderate	Moderate
p21 (CDKN1A) expression, increase (https://aopwiki.org/relationships/1711)	adjacent	Cell cycle, disrupted	High	Moderate
Cell cycle, disrupted (https://aopwiki.org/relationships/1712)	adjacent	Apoptosis	Moderate	Moderate
Apoptosis (https://aopwiki.org/relationships/1735)	adjacent	spermatocyte depletion	High	Not Specified
spermatocyte depletion (https://aopwiki.org/relationships/1734)	adjacent	testicular toxicity	High	Not Specified
Histone deacetylase inhibition (https://aopwiki.org/relationships/1715)	non-adjacent	Cell cycle, disrupted	High	Moderate
Histone deacetylase inhibition (https://aopwiki.org/relationships/1716)	non-adjacent	Apoptosis	Moderate	Moderate
Histone deacetylase inhibition (https://aopwiki.org/relationships/1717)	non-adjacent	testicular toxicity	Moderate	Low

Stressors

Name	Evidence
Methoxyacetic acid	High
Butyrate	High
Trichostatin A	High

Overall Assessment of the AOP

1. Support for Biological Plausibility of KERs	
MIE => KE1: Histone deacetylase inhibition leads to histone acetylation increase	Biological Plausibility of the MIE => KE1 is high. Rationale: Upon the inhibition of HDAC by HDIs, the acetylation of lysine in histone remains and it leads to transcriptional activation or repression, changes in DNA replication and DNA damage repair. (Wade, 2008).
KE1 => KE2: Histone acetylation, increase leads to p21 (CDKN1A) expression, increase	Biological Plausibility of the KE1 => KE2 is moderate. Rationale: HDIs induce histone acetylation increase and p21 expression increase leading to the cell cycle arrest, which suggests the close correlation between histone acetylation increase and p21. In the models proposed for the relationship between histone acetylation and transcription, histone acetylation can be untargeted and occur at both promoter and nonpromoter regions, targeted generally to promoter regions, or targeted to specific promoters by gene-specific activator proteins (Richon, 2000, Struhl, 1998). Since several results supported a model in which increased histone acetylation is targeted to specific genes and occurs throughout the entire gene, not just the promoter regions, histone acetylation may leads to gene transcription of p21 (Richon, 2000).
KE2 => KE3: p21 (CDKN1A) expression, increase leads to cell cycle, disrupted	Biological Plausibility of the KE2 => KE3 is high. Rationale: The study using the p21 deficient lungs showed that p21 is essential for the survival under hyperoxia and protects the lung from oxidative stress (O'Reilly, 2001). Hyperoxia inhibits DNA replication through p21 and histone H3 expression (O'Reilly, 2001). Hyperoxia decreased proliferation in p21 wild-type lungs but not in p21-deficient mice, which suggests that p21 is crucial for cell cycle regulation (O'Reilly, 2001).
KE3 => KE4: Cell cycle disrupted leads to apoptosis	Biological Plausibility of the KE3 => KE4 is high. Rationale: microRNA-497, potentially targeting Bcl2 and Cyclin D2 (CCND2), induced apoptosis via the Bcl-2/Bax - caspase 9 - caspase 3 pathway and CCND2 protein in human umbilical vein endothelial cells (HUVECs) (Wu, 2016). The microRNA-497 activated caspases 9 and 3, and decreased Bcl2 and CCND2 (Wu, 2016). CCND2 is an important cell cycle gene that induces G_1 arrest (Li, 2012), and deregulated CCND2 is implicated in cell proliferation inhibition (Wu, 2016, Mermelstein, 2005, Dong, 2010).
KE4 => KE5: Apoptosis leads to spermatocyte depletion	Biological Plausibility of the KE4 => KE5 is high. Rationale: Apoptosis is a basic biological phenomenon in which the cells are controlled in the atrophy of various tissues and organs through the deletion and turnover, as well as in tumor regression (Kerr, 1972).

KE5 => AO: Spermatocyte depletion leads to testicular toxicity	Biological Plausibility of the KE5 => AO is high. Rationale: HDAC inhibition induced testicular toxicity including testis atrophy [Miller, 1982]. HDAC inhibition in cell culture resulted in the testicular toxicity including germ cell apoptosis and cell morphology change [Li, 1996].
2. Support for essentiality of KEs	
KE4: Apoptosis	Essentiality of the KE4 is moderate. Rationale for Essentiality of KEs in the AOP: HDAC1-defecient embyonic stem cells showed reduced proliferation rates, which correlates with decreased cyclin-associated kinase activities and elevated levels of the cyclin-dependent kinase inhibitor p21 (Lagger, 2002). Loss of HDAC1 leads to significantly reduced overall deacetylase activity, hyperacetylation of a subset of histones H3 and H4 (Lagger, 2002).
3. Empirical support for KERs	
MIE => KE1: Histone deacetylase inhibition leads to histone acetylation increase	Empirical Support of the MIE => KE1 is high. Rationale: HDIs increase histone acetylation in brain (Schroeder, 2013). The HDI selectivity exists, in which SAHA is a more potent inducer of histone acetylation than MS-275, and more acetylation sites on the histones H3 and H4 are responsible to SAHA than MS-275 (Choudhary, 2009). HDI AR-42 induces acetylation of histone H3 in dose-response manner in human pancreatic cancer cell lines (Henderson 2016). To quantify acetylation by HDAC, stable isotope labeling with amino acids in cell culture (SILAC) method is used (Choudhary, 2009). SAHA and MS-275 increased acetylation of specific lysines on histones more than twofold (Choudhary, 2009). Acetylation of the variant histone H2AZ-a mark for DNA damage sites- was upregulated almost 20-fold by SAHA, whereas a number of sites on the core histones H3 and H4 are several times more highly regulated in response to SAHA than by MS-275 (Choudhary, 2009). TSA (100 ng/ml) accumulated the acetylated histones in a variety of mammalian cell lines, and the partially purified HDAC from wild-type FM3A cells was effectively inhibited by TSA (Ki = 3.4 nM) (Yoshida, 1990). To predict the degree of acetylation of lysine, a public database called Phosida (www.phosida.com), which is a member of ProteinEx-change and provides detailed information about each acetylation site is available (Choudhary, 2009, Gnad, 2011). The database contains high-accuracy species-specific phosphorylation and acetylation site predictors and allow the *in silico* determination of modified sites on any protein on the basis of the primary sequence (Gnad, 2011).

KE1 => KE2: Histone acetylation, increase leads to p21 (CDKN1A) expression, increase	Empirical Support of the KE1 => KE2 is moderate. Rationale: Histone acetylation regulates the gene transcriptional mechanism (Struhl, 1998). Histones, which may inhibit RNA synthesis, are acetylated and the acetylation of histones promote the RNA polymerase reaction (Allfrey, 1964, Pogo, 1966). HDIs accumulated acetylation of histones and induced p21 protein and mRNA expression (Richon, 2000, Wu, 2001). TSA (0.3 uM) induced p21 mRNA expression in 1 hr after stimulation and the induction is returned to the basal level in 24 hrs (Wu, 2001). Sodium butyrate (5 mM) and repetitive doses of TSA (0.3 uM, every 8 hrs) induced the p21 mRNA level in 24 hrs in HT-29 cells (Wu, 2001). Time course for histone H4 hyperacetylation in response to in repeated doses of TSA every 8hrs showed that histone hyperacetylation was peaked in 12 hrs in 8-fold increase and showed 5-fold increase in 24 hrs compared to control (Wu, 2001).
KE2 => KE3: p21 (CDKN1A) expression, increase leads to cell cycle, disrupted	Empirical Support of the KE2 => KE3 is high. Rationale: HDIs induce p53-independent expression of p21 via Sp1 binding sites in the p21 promoter (Gartel, 2002). TSA induces p21 expression leading to cell cycle arrest (Gartel, 2002). Butyrate induced p21 and apoptosis in human colon tumor cell lines, whereas the absence of p21 increased the apoptosis in HCT116 colon carcinoma cell line, which indicates that p21 has a repressive effect for butyrate-induced apoptosis and protects the cells from butyrate-induced cell death (Gartel, 2002). SAHA induced p53-independent p21 expression and apoptosis in myelomonocytic leukemia cells (Gartel, 2002). The SAHA-related lethality was increased by anti-sense p21, which indicates a protective role of p21 against SAHA-induced apoptosis (Gartel, 2002). The peptide containing cyclin-binding domain of p21 in N-terminus inhibited the kinase activity of cyclin E-Cdk2 with concentration of inhibitor which inhibits kinase activity to 50% of activity (Ki) of 296 nM (Chen, 1996). The Ki was more than 300,000 nM for inhibition of the kinase activity of cyclin D1-Cdk4, and 220 nM for inhibition of the kinase activity of cyclin A-Cdk2 (Chen, 1996). The peptide containing cyclin-binding domain of p21 in C-terminus showed 32,000, 800, or >300,000 nM of Ki for inhibition of the kinase activity of cyclin E-Cdk2, cyclin A-Cdk2 or cyclin D1-Cdk4, respectively (Chen, 1996). GST fusion proteins of p21 without amino acids 17-24 (cyclin binding site in N-terminus of p21) showed 4.3, 0.4, or >850 nM of Ki for inhibition of the kinase activity of cyclin E-Cdk2, cyclin A-Cdk2, or cyclin D1-Cdk4, respectively (Chen, 1996). Deletion of either cyclin binding site in N-terminus or C-terminus of p21, or CDK binding domain was sufficient for the kinase activity inhibition (Chen, 1996).
KE3 => KE4: Cell cycle disrupted leads to apoptosis	Empirical Support of the KE3 => KE4 is high. Rationale: Cell cycle arrest such as G1 arrest and G1/S arrest are observed in apoptosis (Li, 2012, Dong, 2010). microRNA-1 and microRNA-206 represses CCND2, while microRNA-29 represses CCND2 and induces G1 arrest and apoptosis in rhabdomyosarcoma (Li, 2012). Caspase-3 and caspase-9 activity is measured with the enzyme-catalyzed release of pNA and quantified at 405 nm (Wu, 2016). Apoptosis is measured with Annexin V-FITC probes, and the relative percentage of Annexin V-FITC-positive/PI-negative cells is analyzed by flow cytometry (Wu, 2016).

KE4 => KE5: Apoptosis leads to spermatocyte depletion	Empirical Support of the KE4 => KE5 is high. Rationale: MicroRNA-21 regulates the spermatogonial stem cell homeostasis, in which suppression of microRNA-21 with anti-miR-21 oligonucleotides led to apoptosis of spermatogonial stem cell-enriched germ cell cultures and the decrease in the number of spermatogonial stem cells (Niu, 2011).
KE5 => AO: Spermatocyte depletion leads to testicular toxicity	Empirical Support of the KE5 => AO is high. Rationale: 2-methoxyethanol (ME) or its major metabolite, MAA induced spermatocyte depletion and testicular atrophy [Beattie, 1984].

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Male	High

The AOP is applicable to the reproductively mature males in rats, mice and humans. The administration route or doses of HDAC inhibitors may affect the intensity of the toxicity.

Essentiality of the Key Events

Key Event	Direct/Indirect Evidence
MIE: histone deacetylase inhibition	HDAC inhibition induced testicular toxicity including testis atrophy [Miller, 1982]. HDAC inhibition in cell culture resulted in the testicular toxicity including germ cell apoptosis and cell morphology change [Li, 1996].
KE1: Histone acetylation, increase	The HDAC inhibition induced cell death in spermatocytes in both rat and human seminiferous tubules [Li, 1996].
KE2: p21 (CDKN1A) expression, increase	Loss of HDAC1 in mouse embryonic stem (ES) cells has demonstrated the acetylation of histones H3 and H4, up-regulation of cyclin-dependent kinase inhibitors p21WAF1/CIP1 and p27KIP1 and inhibition of proliferation (Lagger, 2002).
KE3: Cell cycle, disrupted	In HDAC1-/- fibroblast lines, increase in the amount of cells in G1 phase and decrease in the amount of cells in S phase were observed, which indicates the importance of HDAC inhibition in cell cycle regulation [Zupkovitz, 2010].

KE4: Apoptosis	HDAC inhibition leads to cell death through the apoptotic pathways (Falkenberg, 2014).
KE5: spermatocyte depletion	The HDAC inhibition induced cell death in spermatocytes in both rat and human seminiferous tubules [Li, 1996]. The HDAC inhibitor treatment resulted in degeneration in spermatocytes in rat seminiferous tubules [Li, 1996]. The HDAC inhibition induced the germ cell apoptosis in human testicular tissues [Li, 1996].

Weight of Evidence Summary

Biological plausibility, coherence, and consistency of the experimental evidence

The available data supporting the AOP are logic, coherent and consistent with established biological knowledge, whereas there are possibilities for alternative pathways.

Alternative mechanism(s) that logically present themselves and the extent to which they may distract from the postulated AOP

There are some other important apoptotic pathways that are involved in cell death, as well as other important spermatocyte signaling or mechanism influences testicular toxicity.

p53 pathway

The study in which *in vivo* administration of trichostatin A (TSA), a HDI, in mice resulted in male meiosis impairment showed the involvement of p53-noxa-caspase-3 apoptotic pathway in TSA-induced spermatocyte apoptosis [Fenic, 2008]. Other study showed that MAA induced up-regulation of p21 expression is mediated through histone hyperacetylation and independent of p53/p63/p73 [Parajuli, 2014].

NF-kappaB pathway

The present AOP focuses on p21 pathway leading to apoptosis, however, the alternative pathway such as NF-kappaB signaling pathways may be involved in apoptosis of spermatocytes [Wang, 2017].

Communication with Sertoli cells

The present AOP focuses on testicular atrophy by HDAC inhibition-induced apoptosis in spermatocytes, however, the signaling in Setoli cells may be involved in testicular atrophy. Sertoli cell secretes GDNF, FGF2, CXCL12 or Ccl9 molecules, which results in the activation of RET, FGFR, CXCR4 or CCR1 signaling in spermatogonial stem cells, respectively [Chen, 2015].

Decrease in deoxynucleotide pool by MAA

MAA induces decrease in deoxynucleotide pool, resulting apoptosis, which may be an alternative pathway other than p21-mediated pathway [Yamazoe, 2015]. Inhibition of 5,10-CH_2-THF production by MAA may decreases deoxynucleotide pool in spermatocytes [Yamazoe, 2015].

Quantitative Consideration

Concordance of dose-response relationships

This is a quantitative description on dose-response relationships from MIE to AOP. But some KE relationships individually are not fully supported with dose-response relationships, while there is empirical evidence to support that a change in KEup leads to an appropriate change in the respective KEdown.

Temporal concordance among the key events and adverse outcome

Temporal concordance between MIE and AOP has been described with in vivo experimental data. Empirical evidences show temporal concordance between MIE and the individual KEs, however, the temporal concordance among the individual KEs and AO is not fully elucidated.

Strength, consistency, and specificity of association of adverse outcome and initiating event

The scientific evidence on the linkage between MIE and AO has been described.

The quantitative understanding of the AOP in terms of indirect relations between HDAC inhibition and testicular atrophy was examined in in vivo experiments [Foster, 1983, Miller, 1982].

Considerations for Potential Applications of the AOP (optional)

The present AOP can be used in risk assessment of HDAC inhibitors for the anti-cancer drugs in terms of testicular toxicity. HDAC inhibitors nowadays have been utilized as therapeutics for cancer or neurology disease, and the adverse effects of HDAC inhibitors should be evaluated. This AOP elucidating the pathway from HDAC inhibition through testicular toxicity may provides important insights for potential toxicity of HDAC inhibitors. It also provides a basis for the HDAC inhibition-induced epigenetic alteration and cell death. HDAC inhibitors such as rocilinostat are clinically evaluated as anti-cancer drugs in clinical trial.

References

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Richon VM et al. (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci 97:10014-10019

Struhl K. (1998) Histone acetylation and transcriptional regulatory mechanisms. Gene Dev 12:599-606

O'Reilly MA et al (2001) The cyclin-dependent kinase inhibitor p21 protects the lung from oxidative stress. Am J Respir Cell Mol Biol 24: 703-710

Wu R et al. (2016) microRNA-497 induces apoptosis and suppressed proliferation via the Bcl-2/Bax-caspase9-caspase 3 pathway and cyclin D2 protein in HUVECs. PLoS One 11: e0167052

Li L et al. (2012) Downregulation of microRNAs miR-1, -206 and -29 stabilizes PAX3 and CCND2 expression in rhabdomyosarcoma. Lab Invest 92: 571-583

Mermelshtein A et al. (2005) Expression of F-type cyclins in colon cancer and in cell lines from colon carcinomas. Br J Cancer 93: 33

Dong Q et al. (2010) microRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One 5: e10147

Kerr JFR et al. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257

Miller RR et al. (1982) Toxicity of methoxyacetic acid in rats. Fundam Appl Toxicol 2: 158-160

Li LH et al. (1996) 2-Methoxyacetic acid (MAA)-induced spermatocyte apoptosis in human and rat testes: an in vitro comparison. J Androl 17: 538-549

Lagger G et al. (2002) Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 21:2672-2681

Schroeder FA et al. (2013) A selective HDAC 1/2 inhibitor modulates chromatin and gene expression in brain and alters mouse behavior in two mood-related tests. PLoS One 8:e71323

Choudhary C et al. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325:834-840

Henderson SE et al. (2016) Suppression of tumor growth and muscle wasting in a transgenic mouse model of pancreatic cancer by the novel histone deacetylase inhibitor AR-42. Neoplasia 18:765-774

Yoshida M et al. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro trichostatin A. J Biol Chem 265:17174-17179

Gnad F et al. (2011) PHOSIDA 2011: the posttranslational modification database. Nucl Acids Res 39:D253-D260

Allfrey V et al (1964) Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc Natl Acad Sci 51: 786-794

Pogo B et al (1966) RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. Proc Natl Acad Sci 55: 805-812

Richon VM et al. (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci 97:10014-10019

Wu JT et al. (2001) Transient vs prolonged histone hyper acetylation: effects on colon cancer cell growth, differentiation, and apoptosis. Am J Physiol Gastrointest Liver Physiol 280:G482-G490

Gartel AL and Tyner AL (2002) The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 1: 639-649

Chen J et al (1996) Cyclin-binding motifs are essential for the function of p21CIP1. Mol Cell Biol 16: 4673-4682

Li L et al. (2012) Downregulation of microRNAs miR-1, -206 and -29 stabilizes PAX3 and CCND2 expression in rhabdomyosarcoma. Lab Invest 92: 571-583

Dong Q et al. (2010) microRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One 5: e10147

Wu R et al. (2016) microRNA-497 induces apoptosis and suppressed proliferation via the Bcl-2/Bax-caspase9-caspase 3 pathway and cyclin D2 protein in HUVECs. PLoS One 11: e0167052

Niu Z et al. (2011) microRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. Proc Natl Acad Sci 108: 12740-12745

Beattie PJ, et al. (1984) The effect of 2-methoxyethanol and methoxyacetic acid on Sertoli cell lactate production and protein synthesis in vitro. Toxicol Appl Pharmacol 76: 56-61

Zupkovitz G et al. (2010) The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation. Mol Cell Biol 30:1171-1181

Falkenberg KJ and Johnstone RW. (2014) Histone deacetylases and their inhibitors in cancer, neurological disease and immune disorders. Nat Rev Drug Discov 13:673-691

Fenic I et al. (2008) In vivo application of histone deacetylase inhibitor trichostatin-A impairs murine male meiosis. J Andro 29: 172-185

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2:300-312

Wang C et al. (2017) CD147 regulates extrinsic apoptosis in spermatocytes by modulating NFkB signaling pathways. Oncotarget 8: 3132-3143

Chen S and Liu Y. (2015) Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. Reproduction 149: R159-R167

Yamazoe Y. et al. (2015) Embryo- and testicular-toxicities of methoxyacetate and the related: a review on possible roles of one-carbon transfer and histone modification. Food Safety 3:92-107

Foster PM et al. (1983) Testicular toxicity of ethylene glycol monomethyl and monoethyl ethers in the rat. Toxicol Appl Pharmacol 69:385-39

Appendix 1

List of MIEs in this AOP

Event: 1502: Histone deacetylase inhibition (https://aopwiki.org/events/1502)

Short Name: Histone deacetylase inhibition

Key Event Component

Process	Object	Action
enzyme inhibitor activity	histone deacetylase 1	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	MolecularInitiatingEvent
Aop:274 - Histone deacetylase inhibition leads to impeded craniofacial development (https://aopwiki.org/aops/274)	MolecularInitiatingEvent
Aop:275 - Histone deacetylase inhibition leads to neural tube defects (https://aopwiki.org/aops/275)	MolecularInitiatingEvent

Stressors

Name
Methoxyacetic acid
Butyrate
Trichostatin A

Name
Valproic acid
Suberoylanilide hydroxamic acid
MS-275
Apicidin

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

HDIs are classified according to chemical nature and mode of mechanism: the short chain fatty acids (e.g., butyrate, valproate), hydroxamic acids (e.g., suberoylanilide hydroxamic acid or SAHA, Trichostatin A or TSA), cyclic tetrapeptides (e.g., FK-228), benzamides (e.g., N-acetyldinaline and MS-275) and epoxides (depeudecin, trapoxin A) [Richon, 2004, Ropero, 2007, Villar-Garea, 2004]. There is a report showing that TSA and butyrate competitively inhibits HDAC activity [Sekhavat, 2007]. HDIs inhibit preferentially HDACs with some selectiveness [Hu, 2003]. TSA inhibits HDAC1, HDAC3 and HDAC8, whereas MS-27-275 has inhibitory effect for HDAC1 and HDAC3 (IC_{50} value of ~0.2 mM and ~8 mM, respectively), but no effect for HDAC8 (IC_{50} value >10 mM) [Hu, 2003]. TSA inhibits HDAC1, 2, 3 of class I HDACs. HDAC 1, 4, 6 are related to tumor size [Damaskos, 2016]. MAA (2 or 5 mM) inhibited HDAC activity in dose-response manner in rat testis cytosolic and nuclear extracts [Wade, 2008].

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Male	High

The inhibition of HDAC by HDIs is well conserved between species from lower organism to mammals.

- HDIs reduced lethality in *Drosophila* model and the HDAC activity was inhibited with HDIs in rat PC12 cells [Steffan, 2001].
- HDIs inhibited restores the rate of resorption of subretinal blebs in hyper glycemia in brown Norway rat and HDAC activity was inhibited with HDIs in human ARPE19 cells [Desjardins, 2016].
- HDIs were approved as drugs for multiple myeloma and T-cell lymphoma by FDA [Ansari, 2016].
- HDIs inhibited cell growth in human non-small cell lung cancer cell lines [Miyanaga, 2008].
- HDAC acetylation level was increased by HDIs in MRL-lpr/lpr murine model of lupus splenocytes [Mishra, 2003].
- SAHA increased histone acetylation in brain and spleen of mice [Hockly, 2003].
- MAA inhibits HDAC activity in HeLa cells and spleens from C57BL/6 mice [Jansen, 2004].
- It is also reported that MAA inhibits HDAC activity in testis cytosolic and nuclear extract of juvenile rats (27 days old) [Wade, 2008].
- VPA and TSA inhibit HDAC enzymatic activity in mouse embryo and human HeLa cell nuclear extract [Di Renzo, 2007].
- HDAC inhibitors, phenylbutyrate (PB) (2 mM) and TSA (200 nM) acetylate histones H3 and H4 in synovial cells from rats with adjuvant arthritis [Chung, 2003].

Key Event Description

Site of action: The site of action for the molecular initiating event is a cell.

The nucleosome consists of core histones having classes of H2A, H2B, H3 and H4) [Damaskos, 2017]. DNA strand (about 200 bp) wound around the core histones, where histone deacetylase (HDAC) effects on the lysine residue of the histone to hydrolyze the acetyl residue [Damaskos, 2017]. Histone deacetylase inhibitor (HDI) inhibits HDAC and acetylate the histones and release the DNA strand to induce the binding of transcription factors [Taunton, 1996]. HDIs have potentials as anti-cancer pharmaceuticals since HDIs induce the transcriptional restoration of epigenetically silenced tumor suppressor genes by regulating acetylation of histones and non-histone proteins [Lee, 2016] [Minucci, 2006].

It is known that 18 HDAC isoforms are classified into four classes: class I HDACs (isoforms 1, 2, 3, 8), class II isoforms (4, 5, 6, 7, 9, 10) and class III HDACs (the sirtuins) and HDAC11 [Weichert, 2009, Barneda-Zahonero, 2012]. HDACs 1, 2 and 3 are ubiquitously expressed, whereas HDAC8 is predominantly expressed in cells with smooth muscle/myoepithelial differentiation [Weichert, 2009]. HDAC6 is not observed to express in lymphocytes, stromal cells and vascular endothelial cells [Weichert, 2009]. Class III HDACs sirtuins are widely expressed and localized in different cellular compartments [Barneda-Zahonero, 2012]. SirT1 is highly expressed in testis, thymus and multiple types of germ cells [Bell, 2014]. HDAC11 expression is enriched in kidney, brain, testis, heart and skeletal muscle [Barneda-Zahonero, 2012].

Description from EU-ToxRisk deliverable:

Eukaryotic histone deacetylases (HDACs) are grouped, according to phylogeny, into classes 1 through 4 (Gregoretti *et al.*, 2004). The members of groups 1,2 and 4 are dependent on a zinc ion and a water molecule at their active sites, for their deacetylase function. The Sirtuins of class 3 depend on NAD^+, and are considered impervious to most known HDAC inhibitors (Drummond *et al.*, 2005).

Several structurally distinct groups of compounds have been found to inhibit HDACs of class 1, 2 and 4, among others short-chain fatty acids (e.g. butyrate and VPA), hydroxamic acids (e.g. TSA and SAHA) and epoxyketones (e.g. Trapoxin) (Drummond *et al.*, 2005). The hydroxamic acids seem to exert their inhibitory action by mimicking the acetyl-lysine target of HDACs, chelating the zinc ion in the active site and displacing the water molecule (Finnin *et al.*, 1999). Several recent high resolution crystal structures support this mode of inhibition (Decroos *et al.*, 2015; Luckhurst *et al.*, 2016). The mode of inhibition of epoxyketones seems to function the formation of a stable transition state analog with the zinc ion in the active site (Porter and Christianson, 2017). The inhibitory actions of the short-chain fatty acids are less well defined, but it has been speculated that VPA blocks access to the binding pocket (Göttlicher *et al.*, 2001). It has been shown that VPA exert similar gene regulatory effects to TSA, on a panel of migration related transcripts in neural crest cells (Dreser *et al.*, 2015) supporting a mode of action similar to hydroxamic acid type HDAC inhibitors.

How it is Measured or Detected

The measurement of HDAC inhibition monitors the decrease in histone acetylation. The measurement methods include the immunological detection of histone acetylation with anti-acetylated histone antibodies [Richon, 2004]. The histones are isolated from pellets of cells treated with HDIs, followed by acid-urea-triton gel electrophoresis, western blotting, and immunohistochemistry [Richon, 2004]. Epigenetic modifications including the histone acetylation are measured using chromatin immunoprecipitation-microarray hybridization (ChIP-chip) [ENCODE Project Consortium, 2004, Ren, 2004]. ChIP detects physical interaction between transcription factors or cofactors and the chromosome [Johnson, 2007]. The HDAC activity is measured directly with ultra high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) by calculating the ratio of deacetylated peptide and acetylated peptide [Zwick, 2016].

Description from EU-ToxRisk deliverable:

HDAC inhibition can be followed by several different approaches:

-Western blots applying antibodies targeting specific acetylated proteins.

-Commercial fluorimetric and colorimetric kits can be applied to assay HDAC activity from various biological extracts.

References

Damaskos C. et al. (2017) Histone deacetylase inhibitors: an attractive therapeutic strategy against breast cancer. Anticancer Research 37: 35-46.

Taunton J. et al. (1996) A mammalian histone deacetylase related to the Yeast transcriptional regulator Rpd3p. Science 272:408-411.

Lee SC. et al. (2016) Essential role of insulin-like growth factor 2 in resistance to histone deacetylase inhibitor. Oncogene 35:5515-5526.

Minucci S, Pelicci PG. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer. Jan;6(1):38-51.

Weichert W. (2009) HDAC expression and clinical prognosis in human malignancies. Cancer Letters 280:168-176

Barneda-Zahonero B and Parra M (2012) Histone deacetylases and cancer. Mol Oncol 6:579-589

Bell EL et al. (2014) SirT1 is required in the male germ cell for differentiation and fecundity in mice. Development 141:3495-3504

Richon VM et al. (2004) Histone deacetylase inhibitors: assays to assess effectiveness in vitro and in vivo. Methods Enzymol. 376:199-205

The ENCODE Project Consortium. (2004) The ENCODE (ENCyclopedia Of DNA Elements) Project. Science 306:636-640

Ren B and Dynlacht D. (2004) Use of chromatin immunoprecipitation assays in genome-wide location analysis of mammalian transcription factors. Methods Enzymol. 376:304-315

Johnson DS et al. (2007) Genome-wide mapping of in vivo protein-DNA interactions. Science 316:1497-1502

Zwick V et al. (2016) Cell-based multi-substrate assay coupled to UHPLC-ESI-MS/MS for a quick identification of class-specific HDAC inhibitors. J Enzyme Inhib Med Chem 31: 209-214

Steffan JS et al. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature 413:739-743

Desjardins D et al. (2016) Histone deacetylase inhibition restores retinal pigment epithelium function in hyperglycemia. PLoS ONE 11: e0162596

Ansari J et al. (2016) Epigenetics in non-small cell lung cancer: from basics to therapeutics. Transl Lung Cancer Res 5:155-171

Miyanaga A et al. (2008) Antitumor activity of histone deacetylase inhibitors in non-small cell lung cancer cells: development of a molecular predictive model. Mol Cancer Ther 7:1923-1930

Mishra N et al. (2003) Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. J Clin Invest 111: 539-552

Hockly E et al. (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proc Nat Acad Sci 100:2041-2046

Jansen MS et al. (2014) Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition Proc Natl Acad Sci USA 101:7199-7204

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Di Renzo F et al. (2007) Boric acid inhibits embryonic histone deacetylases: A suggested mechanism to explain boric acid-related teratogenicity. Toxicol and Appl Pharmacol 220:178-185

Chung YL et al. (2003) A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. Mol Ther 8:707-717

Ropero S and Esteller M. (2007) The role of histone deacetylases (HDACs) in human cancer. Mol Oncol 1:19-25

Villae-Garea A and Esteller M. (2004) Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int J Cancer 112:171-178

Sekhavat A et al. (2007) Competitive inhibition of histone deacetylase activity by trichostatin A and butyrate. Biochemistry and Cell Biology 85:751-758

Hu E et al. (2003) Identification of novel isoform-selective inhibitors within class I histone deacetylases. J Pharmacol Exp Ther 307:720-728

Damaskos C et al. (2016) Histone deacetylase inhibitors: a novel therapeutic weapon against medullary thyroid cancer? Anticancer Res 36:5019-5024

Decroos, C., Christianson, N.H., Gullett, L.E., Bowman, C.M., Christianson, K.E., Deardorff, M.A., and Christianson, D.W. (2015), Biochemistry 54: 6501–6513.

Dreser, N., Zimmer, B., Dietz, C., Sügis, E., Pallocca, G., Nyffeler, J., et al. (2015), Neurotoxicology 50: 56–70.

Drummond, D.C., Noble, C.O., Kirpotin, D.B., Guo, Z., Scott, G.K., and Benz, C.C. (2005), Annu Rev Pharmacol Toxicol 45: 495–528.

Finnin, M.S., Donigian, J.R., Cohen, a, Richon, V.M., Rifkind, R. a, Marks, P. a, et al. (1999), Nature 401: 188–193.

Göttlicher, M., Minucci, S., Zhu, P., Krämer, O.H., Schimpf, A., Giavara, S., et al. (2001), EMBO J 20: 6969–6978.

Gregoretti, I. V., Lee, Y.M., and Goodson, H. V. (2004), J Mol Biol 338: 17–31.

Luckhurst, C.A., Breccia, P., Stott, A.J., Aziz, O., Birch, H.L., Bürli, R.W., et al. (2016), ACS Med Chem Lett 7: 34–39.

Porter, N.J., and Christianson, D.W. (2017), ACS Chem Biol 12: 2281–2286.

List of Key Events in the AOP

Event: 1503: Histone acetylation, increase (https://aopwiki.org/events/1503)

Short Name: Histone acetylation, increase

Key Event Component

Process	Object	Action
regulation of protein modification process	histone	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	KeyEvent
Aop:275 - Histone deacetylase inhibition leads to neural tube defects (https://aopwiki.org/aops/275)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

The histone acetylation increase by HDIs is well conserved between species from lower organism to mammals.

MAA, a HDAC inhibitor, induces acetylation of histones H3 and H4 in Sprague-Dawley (*Rattus norvegicus)* [Wade, 2008].

It is also reported that MAA promotes acetylation of H4 in HeLa cells (*Homo sapiens*) and spleens from C57BL/6 mice (*Mus musculus*) treated with MAA [Jansen, 2014].

VPA, a HDAC inhibitor, induces hyperacetylation of histone H4 in protein extract of mouse embryos (*Mus musculus*) exposed *in utero* for 1h to VPA [Di Renzo, 2007a].

Apicidin, MS-275 and sodium butyrate, HDAC inhibitors, induce hyperacetylation of histone H4 in homogenates from mouse embryos (*Mus musculus*) after the treatments [Di Renzo, 2007b].

MAA acetylates histones H3K9 and H4K12 in limbs of CD1 mice (*Mus musculus*) [Dayan, 2014].

Key Event Description

Gene transcription is regulated with the balance between acetylation and deacetylation. The acetylation and deacetylation are modulated on the NH_3^+ groups of lysine amino acid residues in histones. DNA in acetylated histones is more accessible for transcription factors, leading to increase in gene expression. HDAC inhibition promotes the hyperacetylation by inhibiting deacetylation of histones with classes of H2A, H2B, H3 and H4 in nucleosomes. [Wade, 2008].

Description from EU-ToxRisk Deliverable:

The inhibition of HDACs result in an accumulation of acetylated proteins such as tubulin or histones.

How it is Measured or Detected

Histone acetylation is measured by the immunological detection of histone acetylation with anti-acetylated histone antibodies [Richon, 2004]. Histone acetylation on chromatin can be measured using labeling method with sodium [^{3}H] acetate [Gunjan, 2001].

Description from EU-ToxRisk Deliverable:

1. Semi-quantitative: Western blot usining antibodies agains acetylated tubulin or histones
2. Quantitative: enzyme assays using acetylated peptides and purified HDAC enzyme

References

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Richon VM et al. (2004) Histone deacetylase inhibitors: assays to assess effectiveness in vitro and in vivo. Methods Enzymol 376:199-205

Gunjan A et al. (2001) Core histone acetylation is regulated by linker histone stoichiometry *in vivo*. J Biol Chem 276:3635-3640

Jansen MS et al. (2014) Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition Proc Natl Acad Sci USA 101:7199-7204

Di Renzo F et al. (2007a) Boric acid inhibits embryonic histone deacetylases: A suggested mechanism to explain boric acid-related teratogenicity. Toxicol and Appl Pharmacol 220:178-185

Di Renzo F et al. (2007b) Relationship between embryonic histonic hyperacetylation and axial skeletal defects in mouse exposed to the three HDAC inhibitors apicidin, MS-275, and sodium butyrate. Toxicol Sci 98:582-588

Dayan C and Hales BF. (2014) Effects of ethylene glycol monomethyl ether and its metabolite, 2-methoxyacetic acid, on organogenesis stage mouse limbs in vitro. Birth Defects Res (Part B) 101:254-261

Event: 1504: p21 (CDKN1A) expression, increase (https://aopwiki.org/events/1504)

Short Name: p21 (CDKN1A) expression, increase

Key Event Component

Process	Object	Action
positive regulation of gene expression	cyclin-dependent kinase inhibitor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

The p21 up-regulation by HDIs is well conserved between species from lower organism to mammals.

FK228, a HDAC inhibitor, up-regulated p21 level in human esophageal cancer TE2 cells (*Homo sapiens*) [Hoshino, 2007].

MAA, a HDAC inhibitor, induced p21 up-regulation in human prostate cancer cell lines (*Homo sapiens*) [Parajuli, 2014].

MAA increases p21 expression in human bladder carcinoma cells, T24 (*Homo sapiens*) [Glaser, 2003].

MAA up-regulated p21 expression in limbs of CD1 embryonic mice (*Mus musculus*) [Dayan, 2014].

Key Event Description

p21 (CDKN1A; cyclin dependent kinase inhibitor 1A) binds to and inhibits the activity of cyclin-dependent kinase 2 or cyclin-dependent kinase 4 complexes, and regulates cell cycle progression in G_1 phase. p21 is important for cell cycle regulation.

How it is Measured or Detected

The p21 mRNA is measured with real-time RT-PCR technique using primers for p21 [Dayan, 2014]. Gene expression of p21 is measured with microarray technique using gene chips after cDNA preparation from total RNA extracted from the samples [Glaser, 2003, Hoshino, 2007]. Protein level of p21 is measured with Western blot analysis using anti-p21 antibody [Parajuli, 2014, Glaser, 2003].

References

Dayan C and Hales BF. (2014) Effects of ethylene glycol monomethyl ether and its metabolite, 2-methoxyacetic acid, on organogenesis stage mouse limbs in vitro. Birth Defects Res (Part B) 101:254-261

Glaser KB et al. (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2:151-163

Hoshino I et al. (2007) Gene expression profiling induced by histone deacetylase inhibitor, FK228, in human esophageal squamous cancer cells. Oncol Rep 18:585-592

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2:300-313

Event: 1505: Cell cycle, disrupted (https://aopwiki.org/events/1505)

Short Name: Cell cycle, disrupted

Key Event Component

Process	Object	Action
regulation of cell cycle	cell cycle-related cyclin	disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between disrupted cell cycle and apoptosis is likely well conserved between species.

The change in the amounts of cells in G_1 phase and S phase of cell cycle was detected in mouse HDAC1 knock out fibroblast lines (*Mus musculus*) [Zupkovitz, 2010].

The histone gene expression alters in each phase of cell cycle in human HeLa cell (*Homo sapiens*) [Heintz, 1982].

Key Event Description

The dysregulation of cell cycle leads to the decreases in cell number. The cell cycle consists of G_1, S, G_2, M, and G_0 phase. The cell cyle regulation is disrupted by the cell cycle arrest in certain cell cycle phase. The histone gene expression is regulated in cell cyle phases [Heintz, 1983]. The phosphorylation of p21 (CDKN1A; cyclin dependent kinase inhibitor 1A) regulates its function [Moussa, 2015, Child, 2006]. The up-regulation of p21 level in iron-chelated cancer cells was observed [Moussa, 2015].

How it is Measured or Detected

The percentage of cells at G_1, G_0, S, and G_2/M phases was determined by flow cytometry analysis using DNA content frequency histogram deconvolution software [Li, 2013].

Cell cycle distribution was analyzed by fluorescence-activated cell sorter (FACS) analysis with a Partec PAS-II sorter [Zupkovitz, 2010].

The four cell cycle phases in living cells can be measured with four-color fluorescent proteins using live cell imaging [Bajar, 2016].

The incorporation of [3H]deoxycytidine or [3H]thymidine into cell DNA during S phase can be monitored as DNA synthesis [Heintz, 1982].

References

Heintz N et al. (1983) Regulation of human histone gene expression: Kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Molecular and Cellular Biology 3:539-550

Moussa RS et al. (2015) Differential targeting of the cyclin-dependent kinase inhibitor, p21CIP/WAF1, by chelators with anti-proliferative activity in a range of tumor cell-types. Oncotarget 6:29694-29711

Child ES and Mann DJ. (2006) The intricacies of p21 phosphorylation. Cell Cycle 5:1313-1319

Li Q, Lambrechts MJ, Zhang Q, Liu S, Ge D, Yin R, Xi M and You Z. Glyphosate and AMPA inhibit cancer cell growth through inhibiting intracellular glycine synthesis. Drug Des Devel Ther 2013; 7: 635-643.

Zupkovitz G et al. (2010) The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation. Mol Cell Biol 30:1171-1181

Bajar BT et al. (2016) Fluorescent indicators for simultaneous reporting of all four cell cycle phases. Nat Methods 13: 993-996

Event: 1262: Apoptosis (https://aopwiki.org/events/1262)

Short Name: Apoptosis

Key Event Component

Process	Object	Action
apoptotic process		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:205 - AOP from chemical insult to cell death (https://aopwiki.org/aops/205)	AdverseOutcome
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans (https://aopwiki.org/aops/207)	KeyEvent
Aop:212 - Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury (https://aopwiki.org/aops/285)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

The apoptosis and proliferation inhibition induced by MAA, a HDAC inhibitor, was measured in human prostate cancer cell lines (*Homo sapiens*) [Parajuli, 2014].

The cell viability inhibition induced by SAHA or TSA , which are HDAC inhibitors, was observed in NHDFs (*Homo sapiens*) [Glaser, 2003].

The proliferation of the HDAC$^{-/-}$ ES cells was inhibited compared to HDAC$^{+/+}$ ES cells (*Homo sapiens*) [Zupkovitz, 2010].

Key Event Description

Apoptosis, the process of programmed cell death, is characterized by distinct morphology with DNA fragmentation and energy dependency [Susan, 2007]. Apoptosis, also called as “physiological cell death”, is involved in cell turnover, physiological involution and atrophy of various tissues and organs [Kerr, 1972]. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances [Kerr, 1972]. Apoptosis is characterized by DNA ladder and chromatin condensation. Several stimuli such as hypoxia, nucleotides deprivation, chemotherapeutical drugs, DNA damage, and mitotic spindle damage induce p53 activation, leading to p21 activation and cell cycle arrest [Pucci, 2000]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation of the NHDF cells [Glaser, 2003]. Considering that the acetylation of histone H4 was increased by the treatment of SAHA for 4 hrs, histone deacetylase inhibition may be involved in the inhibition of the cell proliferation [Glaser, 2003]. The impaired proliferation was observed in HDAC1$^{-/-}$ ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz, 2010]. The present AOP focuses on p21 pathway leading to apoptosis, however, the alternative pathway such as NF-kB signaling pathways may be involved in apoptosis of spermatocytes [Wang, 2017].

How it is Measured or Detected

The apoptosis is detected with the expression alteration of procaspases 7 and 3 by Western blotting using antibodies [Parajuli, 2014].

The apoptosis is measured with down-regulation of anti-apoptotic gene baculoviral inhibitor of apoptosis protein repeat containing 2 (BIRC2, or cIAP1) [Parajuli, 2014].

Apoptotic nucleosomes were detected using Cell Death Detection ELISA kit, which were calculated as absorbance subtraction at 405 nm and 490 nm [Parajuli, 2014].

Cell viability was measured with live cell number changes using the CellTiter-Glo Luminescent Cell Viability Assay [Parajuli, 2014].

Cleavage of PARP was detected with Western blotting [Parajuli, 2014].

The proliferation/viability of NHDFs was measured with Alamar-Blue [modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [Glaser, 2003].

Proliferation of the HDAC$^{-/-}$ ES cells was determined with crystal violet and measurement of absorbance at 595 nm [Zupkovitz, 2010].

Caspase-3 and caspase-9 activity is measured with the enzyme-catalyzed release of p-nitroanilide (pNA) and quantified at 405 nm [Wu, 2016].

Apoptosis is measured with Annexin V-FITC probes, and the relative percentage of Annexin V-FITC-positive/PI-negative cells is analyzed by flow cytometry [Wu, 2016].

Apoptosis is detected with the Terminal dUTP Nick End-Labeling (TUNEL) method to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks [Kressel, 1994].

References

Susan E. (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35: 495-516

Kerr JFR et al. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257

Pucci B et al. (2000) Cell cycle and apoptosis. Neoplasia 2:291-299

Glaser KB et al. (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2:151-163

Zupkovitz G et al. (2010) The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation. Mol Cell Biol 30:1171-1181

Wang C et al. (2017) CD147 regulates extrinsic apoptosis in spermatocytes by modulating NFkB signaling pathways. Oncotarget 8: 3132-3143

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2: 300-313

Wu R et al. (2016) microRNA-497 induces apoptosis and suppressed proliferation via the Bcl-2/Bax-caspase9-caspase 3 pathway and cyclin D2 protein in HUVECs. PLoS One 11: e0167052

Kressel M and Groscurth P (1994) Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA. Cell Tissue Res 278: 549-556

Event: 1515: spermatocyte depletion (https://aopwiki.org/events/1515)

Short Name: spermatocyte depletion

Key Event Component

Process	Object	Action
	spermatocyte	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
testis

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Male	High

There are evidences of spermatocyte depletion.

It has been reported that mice lacking cyclin D-dependent kinase inhibitor proteins produced few mature sperm, and the residual spermatozoa had reduced motility and decreased viability (*Mus musculus*) [Zindy, 2001].

The sperm counts in the cauda epidydimis of rats exposed to butylparaben were significantly decreased (*Rattus norvegicus*) [Oishi, 2001].

MAA treatment induced spermatocyte death in Sprague-Dawley rats (*Rattus norvegicus*) [Wade, 2008].

Key Event Description

The apoptosis of the cells lead to spermatocyte depletion. Spermatocytes are differentiated from spermatogonial stem cells via random proliferation, differentiation and synchronized mitoses with several stages [Rooij, 2001].

How it is Measured or Detected

The sperm-containing fluid was squeezed out of the cauda, and suspended in medium containing HEPEs buffer and bovine serum albumin, and incubated at 37C for 20 min. The number of spermatozoa was determined by hematocytometer [Zindy, 2001].

Testicular sperm counts and daily sperm production were determined by counting the total number of spermatids per testis, and divided by the testicular weight to give the results in spermatids per gram of testis [Oishi, 2001].

For the detection of apoptosis, the testes were fixed in neutral buffered formalin, and embedded in paraffin. Germ cell death was visualized in testis sections by Terminal dUTP Nick End-Labeling (TUNEL) staining memthod [Wade, 2008]. The incidence of TUNEL-positive cells was expressed as the number of positive cells per tubule examined for one entire testis section per animal [Wade, 2008].

For the testis cell analysis, fresh testes were dispersed using a two-stage enzymatic digestion and incubated in BSA containing collagenase and DNase I [Wade, 2006]. The seminiferous tubules were further digested and cells were fixed in ice-cold 70% ethanol [Wade, 2006]. Relative proportions of spermatogenic cell populations were assessed in fixed cells using a flow cytometeric method [Wade, 2006]. The principle of the test is that spermatogenic cells, as they differentiate from normal diploid spermatogonial stem cells through to mature spermatozoa with a highly condensed haploid complement of DNA, progress through various intermediate stages with differing nuclear DNA content and cellular content of mitochondria. Relative proportions of cells in each population were calculated with WinList software [Wade, 2006].

References

Rooij DG. (2001) Proliferation and differentiation of spermatogonial stem cells. Reproduction 121:347-354

Zindy F et al. (2001) Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors $p18^{Ink4c}$ and $p19^{Ink4d}$. Mol Cell Biol 21:3244-3255

Oishi S. (2001) Effects of butylparaben on the male reproductive system in rats. Toxicol Indust Health 17:31-39

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Wade MG et al. (2006) Testicular toxicity of candidate fuel additive 1,6-dimethoxyhexane: comparison with several similar aliphatic ethers. Toxicol Sci 89:304-313

List of Adverse Outcomes in this AOP

Event: 1506: testicular toxicity (https://aopwiki.org/events/1506)

Short Name: testicular toxicity

Key Event Component

Process	Object	Action
testicular atrophy	Testis	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	AdverseOutcome

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
testis

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Male	High

There are some evidences on testicular toxicity induced by HDAC inhibitors.

EGME or MAA treatment induced the testicular damage in rat (*Rattus norvegicus*) [Foster, 1983].

EGME were shown to deplete the spermatocytes in CD-1 mice (*Mus musculus*) and CD rats (*Rattus norvegicus*), principally pachytene cells, but with other stages affected with increasing dose [Anderson, 1987].

The testicular lesions induced by 2-methoxyethanol were observed in rats (*Rattus norvegicus*) and guinea pigs (*Cavia porcellus*), which are different in onset, characteristics and severity [Ku, 1984].

EGME has effects in disruption of spermatogenesis in rabbits (*Oryctolagus cuniculus*) [Foote, 1995].

Dimethoxyhexane (DMH) induces testicular toxicity such as spermatocyte death in seminiferous tubule stages I-IV and stages XII-XIV and MAA increase in urine in Sprague-Dawley rats (*Rattus norvegicus*).

Key Event Description

It is hypothesized that the testicular effects of 1,6-dimethoxyhexane (DMH) are caused by its metabolism to MAA [Wade, 2006, Poon, 2004]. MAA produces testicular and thymic atrophy such as the decrease in size [Miller, 1982, Moss, 1985]. The spermatogenic stages in which the toxicity of MAA is induced are on the patchytene spermatocytes immediately before and during meiotic division, which are Stages XII-XIV of the cycle in the rat and the early pachytene spermatocytes at stages I-IV of the cycle. Dead germ cells can be seen as soon as 12 hours after the treatment of MAA [Casarett & Doull's 7th edition].

How it is Measured or Detected

The weights of testes of MAA-treated rats were measured to detect the testicular atrophy [Foster, 1983]. Since zinc concentration has been

shown to play an important role in the production of testicular injury by compounds, the effects of EGME and MAA on urinary zinc excretion and testicular zinc content was examined [Foster, 1983]. Testis were fixed for observations for light microscopy or transmission electron microscopy [McDowell, 1976, Mercantepe, 2018]. The testicular tissue structure was observed whether there are normal germinal epithelial cells and Leydig cells [Mercantepe, 2018]. Changes in sperm were measured by computer-assisted sperm analysis [Foote, 1995].

For the assessment of sperm morphology, eosin-stained sperm collected from the cauda epididymis were smeared onto two glass slides per sample, air-dried, and cover-slipped. At least 200 sperm on each slide were examined for the proportion of sperm with abnormal head (overhooked, blunt hook, banana-shaped, amorphous, or extremely oversized) or tail (twisted, bent, corkscrew, double. multiple) by one individual unaware of animal number or treatment [Wade, 2006]. For the measurement of the total number of condensed spermatids per testis, a weighed portion of the parenchyma from the left testis, as representative of the whole organ as possible, was homogenized [Wade, 2006]. For the measurement of the total number of sperm in the cauda epididymis, whole cauda and associated sperm suspension in DPBS were thawed on ice and homogenized [Wade, 2006]. Sperm or homogenization-resistant spermatid nuclei densities were calculated from the average number of nuclei and were expressed as total or as per gram of epididymis or testis weight [Wade, 2006]. For the determination of total LDH and LDH-X in supernatant of the homogenized testis fragment, enzyme activity was measured by monitoring extinction of NAD absorbance [Wade, 2006].

Regulatory Significance of the AO

The testicular toxicity assessment is important for assessing the side effects of the medicines such as anti-cancer drugs. The unexpected effects may be predicted with this AO.

References

Wade MG et al. (2006) Testicular toxicity of candidate fuel additive 1,6-dimethoxyhexane: comparison with several similar aliphatic ethers. Toxicol Sci 89:304-313

Poon R et al. (2004) Short-term oral toxicity of pentyl ether, 1,4-diethoxybutane, and 1,6-dimethoxyhexane in male rats. Toxicol Sci 77:142-150

Miller R et al. (1982) Toxicity of methoxyacetic acid in rats. Fundam Appl Toxicol 2:158-160

Moss EJ et al. (1985) The role of metabolism in 2-methoxyethanol-induced testicular toxicity. Toxicol Appl Pharmacol 79:480-489

Casarett & Doull's Toxicology, the Basic Science of Poisons, 7th Edition, Edited by Curtis D. Klaassen, Chapter 20 Toxic responses of the reproductive system

Foster PM et al. (1983) Testicular toxicity of ethylene glycol monomethyl and monoethyl ethers in the rats. Toxicol Appl Pharmacol 69:385-399

McDowell EM and Trump BF. (1976) Histologic fixatives suitable for diagnostic light and electron microscopy. Arch Pathol Lab Med 100:405-414

Mercantepe T et al. (2018) Protective effects of amifostine, curcumin and caffeic acid phenethyl ester against cisplatin-induced testis tissue damage in rats. Exp Ther Med 15:3404-3412

Foote RH et al. (1995) Ethylene glycol monomethyl ether effects on health and reproduction in male rabbits. Reprod Toxicol 9:527-539

Anderson D et al. (1987) Effect of ethylene glycol monomethyl ether on spermatogenesis, dominant lethality, and F1 abnormalities in the rat and the mouse after treatment of F0 males. Teratog Carcinog Mutagen 7:141-158

Ku WW et al. (1994) Comparison of the testicular effects of 2-methoxyethanol (ME) in rats and guinea pigs. Exp Mol Pathol 61:119-133

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1709: Histone deacetylase inhibition leads to Histone acetylation, increase (https://aopwiki.org/relationships/1709)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**adjacent**	**High**	**Moderate**

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leads to neural tube defects (https://aopwiki.org/aops/275)	**adjacent**	**Not Specified**	**Not Specified**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Oryctolagus cuniculus	Oryctolagus cuniculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9986)
Brassica napus	Brassica napus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=3708)

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between HDAC inhibition and hyperacetylation is likely well conserved between species from lower organisms to mammals.

- Hyperacetylation by HDIs such as SAHA and Cpd-60 are observed in mouse (*Mus musculus*) [Schroeder, 2013].
- TSA induces acetylation of histone H4 in time-dependent manner in mouse cell lines (*Mus musculus*) [Alberts, 1998].
- AR-42, a novel HDI, induces hyperacetylation in human pancreatic cancer cells (*Homo sapiens*) [Henderson, 2016].
- SAHA and MS-275 hyperacetylates lysine of histones in human cell lines of epithelial (A549) and lymphoid origin (Jurkat) (*Homo sapiens*) [Choudhary, 2009].
- SAHA treatment induces the H3 and H4 histone acetylation in human corneal fibroblasts and conjunctiva from rabbits after glaucoma filtration surgery (*Homo sapiens*, *Oryctolagus cuniculus*) [Sharma, 2016].
- TSA induces the acetylation of histones H3 and H4 in *Brassica napus* microspore cultures (*Brassica napu*) [Li, 2014].

Key Event Relationship Description

The HDAC inhibitors (HDIs) inhibit deacetylation of the histone, leading to the increase in histone acetylation and gene transcription. HDACs deacetylate acetylated histone in epigenetic regulation [Falkenberg, 2014].

Description from EU-ToxRisk Deliverable:

Histone acetylation is one of the major epigenetic mechanisms and belongs to the posttranslational modifications of histones. Histone acetyltransferases are setting the mark and deacetylases (HDAC) are responsible for removing the acetyl group from specific lysin residues of the histones. It has been shown that the inhibition of HDACs leads to a hyperacetylation of histones and in general to an imbalance of other histone modifications.

Evidence Supporting this KER

Biological Plausibility

HDACs are important proteins in epigenetic regulation of gene transcription. Upon the inhibition of HDAC by HDIs, the acetylation of lysine in histone remains and it leads to transcriptional activation or repression, changes in DNA replication and DNA damage repair. The treatment by HDIs increased histone acetylation [Wade, 2008].

Description from EU-ToxRisk Deliverable:

In all eukaryotes the DNA containing the genetic information of an organism, is organized in chromatin. The basic unit of chromatin is the nucleosome around which the naked DNA is wrapped. A nucleosome consists of two copies of each of the core histones H2A, H2B, H3 and H4 (Luger et al., 1997). In order to dynamically regulate this highly complex structure severeal mechanism are involved, including the posttranslational modifications of histones (reviewed in (Bannister and Kouzarides, 2011; Kouzarides, 2007). For long time it is known that histones get acetylated and that this reaction is catalyzed by histone acetyl transferases (HAT) and the acetyl groups are removed by histone deacetylases (HDAC). Inhibition of HDACs by small molecule compounds lead to hyperacetylation of the histones as the homeostasis of acetylation and deacetylation is disturbed (reviewed in (Gallinari et al., 2007)).

Empirical Evidence

- HDAC inhibition by HDIs leads to hyperacetylation of histone and a large number of cellular proteins such as NF-kB [Falkenberg, 2014, Chen, 2018].
- The concentrations of half-maximum inhibitory effect (IC_{50}) for HDAC enzyme inhibition of 20 valproic acid derivatives correlated with teratogenic potential of the compounds, and HDAC inhibitory compounds showed hyperacetylation of core histone 4 in treated F9 cells [Eikel, 2006].
- HDIs increase histone acetylation in brain [Schroeder, 2013].
- The HDI selectivity exists, in which SAHA is a more potent inducer of histone acetylation than MS-275, and more acetylation sites on the histones H3 and H4 are responsible to SAHA than MS-275 [Choudhary, 2009].
- HDI AR-42 induces acetylation of histone H3 in dose-response manner in human pancreatic cancer cell lines [Henderson, 2016].
- MAA treatment in rats (650 mg/kg, for 4, 8, 12, and 24 hrs) induced hyperacetylation in histones H3 and H4 of testis nuclei [Wade, 2008].
- HDAC inhibition induced by valproic acid (VPA) leads to histone hyperacetylation in mouse teratocarcinoma cell line F9 [Eikel, 2006].
- Hyperacetylation of histone H3 was observed in HDAC1-deficient ES cells [Lagger, 2002].
- The treatment of MAA induced histone acetylation in H3K9Ac and H4K12Ac, as well as p53K379Ac [Dayan, 2014].

Description from EU-ToxRisk Deliverable:

The major empirical evidence came from the incubation of cell culture cells with small molecule compounds that inhibit HDAC enzymes followed by western blots or acid urea gel analysis.

The first evidence was shown by Riggs et al. who showed that incubation of HeLa cells with n-butyrate leads to an accumulation of acetylated histone proteins (Riggs et al., 1977)

Later, it was shown that n-butyrate specifically increases the acetylation of histone by the incorporation of radioactive [H^3] acetate and analysis of the histones on acid urea gels that allow the detection of acetylated histones (Cousens et al., 1979)

TSA was shown to be an HDAC inhibitor by acid urea gel analysis in 1990 (Yoshida et al., 1990) and good evidence for VPA as an HDAC inhibitor in vitro and in vivo was shown using acetyl-specific antibodies and western blot (Gottlicher et al., 2001).

Exposure of mouse embryos in utero to VPA and TSA (two well-known HDAC inhibitors) showed an increased histone acetylation level in whole embryo extracts and was also shown in situ immuno stainings (Menegola et al., 2005).

Uncertainties and Inconsistencies

HDACs affect a large number of cellular proteins including histones, which reminds us the HDAC inhibition by HDIs hyperacetylates cellular proteins other than histones and exhibit biological effects. It is also noted that HDAC functions as the catalytic subunits of large protein complex, which suggests that the inhibition of HDAC by HDIs affect the function of the large multiprotein complexes of HDAC [Falkenberg, 2014].

Description from EU-ToxRisk Deliverable:

All above mentioned analysis are indirect or in purified systems. Therefore a direct cause-consequence relation is difficult to obtain.

Quantitative Understanding of the Linkage

To quantify acetylation by HDAC, stable isotope labeling with amino acids in cell culture (SILAC) method is used [Choudhary, 2009].

Response-response relationship

SAHA and MS-275 increased acetylation of specific lysines on histones more than twofold [Choudhary, 2009]. Acetylation of the variant histone H2AZ-a mark for DNA damage sites- was upregulated almost 20-fold by SAHA, whereas a number of sites on the core histones H3 and H4 are several times more highly regulated in response to SAHA than by MS-275 [Choudhary, 2009].

TSA (100 ng/ml) accumulated the acetylated histones in a variety of mammalian cell lines, and the partially purified HDAC from wild-type FM3A cells was effectively inhibited by TSA (K_i = 3.4 nM) [Yoshida, 1990].

References

Falkenberg KJ and Johnstone RW. (2014) Histone deacetylases and their inhibitors in cancer, neurological disease and immune disorders. Nat Rev Drug Discov 13:673-691Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Chen S et al. (2018) Valproic acid attenuates traumatic spinal cord injury-induced inflammation via STAT1 and NF-kB pathway dependent of HDAC3. J Neuroinflammation 15:150

Eikel D et al. (2006) Teratogenic effects mediated by inhibition of histone deacetylases: evidence from quantitative structure activity relationships of 20 valproic acid derivatives. Chem Res Toxicol 19:272-278

Schroeder FA et al. (2013) A selective HDAC 1/2 inhibitor modulates chromatin and gene expression in brain and alters mouse behavior in two mood-related tests. PLoS One 8:e71323

Choudhary C et al. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325:834-840

Henderson SE et al. (2016) Suppression of tumor growth and muscle wasting in a transgenic mouse model of pancreatic cancer by the novel histone deacetylase inhibitor AR-42. Neoplasia 18:765-774

Lagger G et al. (2002) Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 21:2672-2681

Dayan C and Hales BF. (2014) Effects of ethylene glycol monomethyl ether and its metabolite, 2-methoxyacetic acid, on organogenesis stage mouse limbs in vitro. Birth Defects Res (Part B) 101:254-261

Yoshida M et al. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro trichostatin A. J Biol Chem 265:17174-17179

Alberts AS et al. (1998) Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyperacetylation. Cell 92:475-487

Sharma A et al. (2016) Epigenetic modification prevents excessive wound healing and scar formation after glaucoma filtration surgery. Invest Ophthalmol Vis Sci 57:3381-3389

Li H et al. (2014) The histone deacetylase inhibitor trichostatin A promotes totipotentcy in the male gametophyte. Plant Cell 26:195-209

Bannister, A. J. and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Res 21*, 381-395. doi:10.1038/cr.2011.22

Cousens, L. S., Gallwitz, D. and Alberts, B. M. (1979). Different accessibilities in chromatin to histone acetylase. *J Biol Chem 254*, 1716-1723.

Gallinari, P., Di Marco, S., Jones, P. et al. (2007). Hdacs, histone deacetylation and gene transcription: From molecular biology to cancer therapeutics. *Cell Res 17*, 195-211. doi:7310149 [pii]

10.1038/sj.cr.7310149

Gottlicher, M., Minucci, S., Zhu, P. et al. (2001). Valproic acid defines a novel class of hdac inhibitors inducing differentiation of transformed cells. *Embo J 20*, 6969-6978. doi:10.1093/emboj/20.24.6969

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell 128*, 693-705.

Luger, K., Mader, A. W., Richmond, R. K. et al. (1997). Crystal structure of the nucleosome core particle at 2.8 a resolution. *Nature 389*, 251-260. doi:10.1038/38444

Menegola, E., Di Renzo, F., Broccia, M. L. et al. (2005). Inhibition of histone deacetylase activity on specific embryonic tissues as a new mechanism for teratogenicity. Birth Defects Res B Dev Reprod Toxicol 74, 392-398. doi:10.1002/bdrb.20053

Riggs, M. G., Whittaker, R. G., Neumann, J. R. et al. (1977). N-butyrate causes histone modification in hela and friend erythroleukaemia cells. *Nature 268*, 462-464.

Yoshida, M., Kijima, M., Akita, M. et al. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin a. *J Biol Chem 265*, 17174-17179.

Relationship: 1710: Histone acetylation, increase leads to p21 (CDKN1A) expression, increase (https://aopwiki.org/relationships/1710)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between increased histone acetylation and p21 expression increase is likely well conserved between species.

- TSA and sodium butyrate induced p21 mRNA expression in HT-29 human colon carcinoma cells (*Homo sapiens*) [Wu, 2001].
- VPA increased acetylation of histone H3 from 3 hrs to 72 hrs after the treatment, and increased p21 expression in 24 hrs after the treatment in K562 cells (*Homo sapiens*) [Gurvich, 2004].
- Scriptaid, a HDI, up-regulated p21 mRNA expression in mouse embryonic kidney cells (*Mus musculus*) [Chen, 2011].

Key Event Relationship Description

Upon histone acetylation increase, p21 transcription and protein level are increased. Acetylation of p21 promoter and p21 mRNA level have a close correlation [Gurvich, 2004]. Transient histone hyperacetylation was sufficient for the activation of p21 [Wu, 2001]. Histone hyperacetylating agents butyrate and TSA induced p21 mRNA expression [Archer, 1998]. SAHA induced the accumulation of acetylated histones in the chromatin of the $p21^{WAF1}$ gene and this increase was associated with an increase in $p21^{WAF1}$ expression [Richon, 2000].

Evidence Supporting this KER

Biological Plausibility

HDIs induce histone hyperacetylation and p21 activation leading to the cell cycle arrest, which suggests the close correlation between histone hyperacetylation and p21. In the models proposed for the relationship between histone acetylation and transcription, histone acetylation can be untargeted and occur at both promoter and nonpromoter regions, targeted generally to promoter regions, or targeted to specific promoters by gene-specific activator proteins [Richon, 2000, Struhl, 1998]. Since several results supported a model in which increased histone acetylation is targeted to specific genes and occurs throughout the entire gene, not just the promoter regions, histone acetylation may leads to gene transcription of p21 [Richon, 2000].

Empirical Evidence

- MAA induced histone acetylation of H4 in prostate cancer cells including LNCaP, C4-2B, PC-3 and DU-145 parallel with p21 mRNA level increase [Parajuli, 2014].
- HDIs accumulated acetylation of histones and induced p21 protein and mRNA expression [Richon, 2000, Wu, 2001].

Uncertainties and Inconsistencies

There are several pathways to activate p21 promoter by HDI. A HDI, apicidin, induced $p21^{WAF1/Cip1}$ mRNA independent of the *de novo* protein synthesis and activated the $p21^{WAF1/Cip1}$ promoter through Sp1 sites [Han, 2001]. Pretreatment with selective PKC inhibitors calphostin A and rottlerin suppressed the promoter activity of p21WAF1/Cip1 activated by apicidin [Han, 2001]. Furthermore, apicidin-induced translocation of PKCe from cytosolic to particulate fraction was reversed by pretreatment with calphostin C, which suggests the PKCe involvement in apicidin-induced $p21^{WAF1/Cip1}$ transcription [Han, 2001]. The p21 promoter activation through Sp1 sites induced by apicidin is thought to be independent of histone hyperacetylation [Han, 2001]. The apicidin is suggested to histone hyperacetylation leading to the antiproliferative activity [Han, 2000]. These results indicate the inconclusive discussion in the linkage between histone acetylation and p21 activation.

Quantitative Understanding of the Linkage

Histone H4 acetylation is induced with in 4 hrs and returned to basal level after 0.3 uM of trichostatin A (TSA) treatment [Wu JT].

Response-response relationship

Dose-response of valproic acid (VPA) showed that 5, 10, and 20 mM of VPA inhibited HDAC6 and HDAC7 activity in 293T cells, and 0.1-2 mM of VPA induced acetylation of lysine in H3 in U937 cells [Gurvich 2004]. The p21 protein level was induced with the treatment of 0.25-2 mM of VPA in U937 cells [Gurvich 2004].

Time-scale

Time course for histone H4 hyperacetylation in response to in repeated doses of TSA every 8 hrs showed that histone hyperacetylation was peaked in 12 hrs in 8-fold increase and showed 5-fold increase in 24 hrs compared to control [Wu JT]. TSA (0.3 uM) induced p21 mRNA expression in 1 hr after stimulation and the induction is returned to the basal level in 24 hrs [Wu JT]. Sodium butyrate (5 mM) and repetitive doses of TSA (0.3 uM, every 8 hrs) induced the p21 mRNA level in 24 hrs in HT-29 cells [Wu JT]. Acetylation of p21 promoter and p21 mRNA induction were correlated in treatment of valproic acid and analogs [Gurvich 2004]. MAA-induced acetylation increase in histones H3 and H4 was occurred in 4, 8, 12 hrs and returned to basal level in 24 hrs after the treatment in rat testis [Wade 2008].

References

Gurvich N et al. (2004) Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 64:1079-1086

Wu JT et al. (2001) Transient vs prolonged histone hyper acetylation: effects on colon cancer cell growth, differentiation, and apoptosis. Am J Physiol Gastrointest Liver Physiol 280:G482-G490

Archer SY et al. (1998) p21^{WAF1} is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc Natl Acad Sci USA 95:6791-6796

Richon VM et al. (2000) Histone deacetylase inhibitor selectively induces p21^{WAF1} expression and gene-associated histone acetylation. Proc Natl Acad Sci 97:10014-10019

Struhl K. (1998) Histone acetylation and transcriptional regulatory mechanisms. Gene Dev 12:599-606

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2:300-313

Han JW et al. (2001) Activation of p21$^{WAF1/Cip1}$ transcription through Sp1 sites by histone deacetylase inhibitor apicidin: involvement of protein kinase C. J Biol Chem 276:42084-42090

Han JW et al. (2000) Apidin, a histone deacetylase inhibitor, inhibits proliferation of tumor cells via induction of p21$^{WAF1/Cip1}$ and gelsolin. Cancer Res 60:6068-6074

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Chen S et al (2011) Histone deacetylase (HDAC) activity for embryonic kidney gene expression, growth, and differentiation. J Biol Chem 286: 32775-32789

Relationship: 1711: p21 (CDKN1A) expression, increase leads to Cell cycle, disrupted (https://aopwiki.org/relationships/1711)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**adjacent**	**High**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

DNA replication in *Xenopus* was suppressed by the GST fusion protein of p21 without amino acids 17-24 or the peptide containing cyclin binding site in N-terminus of p21 protein [Chen, 1996]. P21 regulates the E2F transcriptional activity to control cell cycle in human U2OS osteosarcoma cells (*Homo sapiens*) [Delavaine, 1999]. Cell cycle is regulated by p21 through cyclins and CDKs in mice (*Mus musculus*) [Sherr CJ, 2004].

Key Event Relationship Description

Cell cycle regulation through p21 (cyclin dependent kinase inhibitor 1A; CDKN1A) activation is demonstrated by the interactions of p21 with cyclins [Dotto, 2000]. p21 interacts directly with cyclins through a conserved region in close to its N-terminus (amino acids 17-24; Cy1) [Dotto, 2000]. The cyclin dependent kinase inhibitor, p21 has the secondary weak cyclin binding domain near its C-terminus region (amino acids 153-159),

which overlaps with its proliferating cell nuclear antigen (PCNA) binding domain [Dotto, 2000]. Kinase activity of cyclin-dependent kinase (Cdk) was inhibited by Cy1 site of p21 that is important for the interaction of p21 with cyclin-Cdk complexes [Chen, 1996]. The p21 inhibits Cdk complexes such as cyclin A/E-Cdk2 or cyclin D-Cdk4 complexes, leading to the cell cycle disruption as G_1/S arrest [Chen, 1996].

Evidence Supporting this KER

Biological Plausibility

p21 has a separate cyclin-dependent kinase 2 (CDK2) binding site in its N-terminus region (amino acids 53-58) and optimal cyclin/CDK inhibition requires binding by this site as well as one of the cyclin binding sites [Dotto, 2000]. The peptide containing Cy1 site inhibited the kinase activity of cyclin E-Cdk2 and cyclin A-Cdk2 [Chen, 1996]. The p21$^{WAF1/CIP1/sdi1}$ gene product inhibits the cyclin D/cdk4/6 and the cyclin E/cdk2 complexes in response to DNA-damage, resulting in G_1/S arrest [Moussa, 2015, Ogryzko, 1997]. p21 inhibits cyclin-dependent kinases and regulates cell cycle to promote cell cycle arrest. Deletion of either cyclin binding site in N-terminus or C-terminus of p21, or CDK binding domain was sufficient for the kinase activity inhibition [Chen, 1996].

Empirical Evidence

- TSA induces p21 expression leading to cell cycle arrest [Gartel, 2002].
- The up-regulation of p21 signaling and in testicular germ cells was observed in diabetes [Kilarkaje, 2015].
- A study investing the effects of miR-6734 that has a sequence homology with a specific region of p21$^{WAF1/CIP1}$ promoter on HCT-116 colon cancer cell growth indicated that miR-6734 up-regulated p21 gene expression and induced cell cycle arrest [Kang, 2016]. This result suggests that the direct enhancement of p21 gene expression is related to the alteration of the cell cycle distribution [Kang, 2016].
- The study of postnatal telomere indicated that dysfunction of premature telomere induces cell-cycle arrest through p21 activation in mammalian cardiomyocytes [Aix, 2016].
- The p21$^{WAF1/CIP1/sdi1}$ gene product inhibits the cyclin D/cdk4/6 and the cyclin E/cdk2 complexes in response to DNA-damage, resulting in G_1/S arrest [Moussa, 2015, Ogryzko, 1997].

Uncertainties and Inconsistencies

TSA promotes apoptosis via HDAC inhibition and p53 signaling pathway activation [Deng, 2016a]. It is suggested that furazolidone induces reactive oxygen species leading to suppression of p-AKT and p21, and induction of apoptosis [Deng, 2016b]. The dual roles of p21 in cell cycle arrest and anti-apoptotic effect in the testicular germ cells of diabetic rats are suggested [Kilarkaje, 2015]. The anti-apoptotic effect of p21 is mediated by caspase-3 inhibition, which demonstrates the possibility of cell-cycle independent effect on apoptosis [Deng, 2016b]. It has been demonstrated that p21 induces apoptosis in human cervical cancer cell lines [Tsao, 1999], whereas p21 is implicated in apoptosis inhibition by blocking activation of caspase-3 or interacting with ASK1 [Gartel, 2002, Zhan, 2007]. Up-regulation of p21 is implicated in the activation of DNA damage pathways, and deletion of p21 improved stem cell function and lifespan without accelerating chromosomal instability, which indicates that p21-dependent checkpoint induction affects the longevity limit [Choudhury, 2007].

Quantitative Understanding of the Linkage

Response-response relationship

The peptide containing cyclin-binding domain of p21 in N-terminus inhibited the kinase activity of cyclin E-Cdk2 with 296 nM of the concentration in which kinase activity is inhibited in 50% (Ki) [Chen, 1996].

The peptide containing cyclin-binding domain of p21 in C-terminus showed 32,000, 800, or >300,000 nM of Ki for inhibition of the kinase activity of cyclin E-Cdk2, cyclin A-Cdk2 or cyclin D1-Cdk4, respectively [Chen, 1996].

References

Dotto GP (2000) p21$^{WAF1/Cip1}$: more than a break to the cell cycle? Biochim Biophys Acta 1471: M43-M56

Chen J et al (1996) Cyclin-binding motifs are essential for the function of p21^{CIP1}. Mol Cell Biol 16: 4673-4682

Moussa RS et al. (2015) Differential targeting of the cyclin-dependent kinase inhibitor, p21CIP/WAF1, by chelators with anti-proliferative activity in a range of tumor cell-types. Oncotarget 6:29694-29711

Ogryzko VV et al. (1997) WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. Mol Cell Biol 17:4877-4882

Gartel AL and Tyner AL (2002) The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 1: 639-649

Kilarkaje N and Al-Bader MM. (2015) Diabetes-Induced Oxidative DNA Damage Alters p53-p21$^{CIP1/Waf1}$ Signaling in the Rat Testis. *Reproductive Sciences* 22: 102–112

Kang MR et al (2016) miR-6734 up-regulates p21 gene expression and induces cell cycle arrest and apoptosis in colon cancer cells. PLoS One 11: e0160961

Aix E et al (2016) Postnatal telomere dysfunction induces cardiomyocyte cell-cycle arrest through p21 activation. J Cell Biol 213: 571-583

Deng Z et al. (2016a) Histone deacetylase inhibitor trichostatin A promotes the apoptosis of osteosarcoma cells through p53 signaling pathway activation. Int J Biol Sci 12:1298-1308

Deng S et al (2016b) P21$^{Waf1/Cip1}$ plays a critical role in furazolidone-induced apoptosis in HepG2 cells through influencing the caspase-3 activation and ROS generation. Food Chem Toxicol 88: 1-12

Tsao YP et al (1999) Adenovirus-mediated p21$^{WAF1/SDII/CIP1}$ gene transfer induces apoptosis of human cervical cancer cell lines. J Virology 73: 4983-4990

Zhan J et al (2007) Negative regulation of ASK1 by p21Cip1 involves a small domain that includes serine 98 that is phosphorylated by ASK1 in vivo. Mol Cell Biol 27: 3530-3541

Choudhury AR et al (2007) Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. Nat Genet 39: 99-105

Delavaine L and La Thangue NB (1999) Control of E2F activity by p21Waf1/Cip1. Oncogene 18: 5381-5392

Sherr CJ and Roberts JM (2004) Living with or without cyclins and cyclin-dependent kinases. Gene Dev 18: 2699-2711

Relationship: 1712: Cell cycle, disrupted leads to Apoptosis (https://aopwiki.org/relationships/1712)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Oryctolagus cuniculus	Oryctolagus cuniculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9986)

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between disrupted cell cycle and apoptosis is likely well conserved between species.

- MicroRNA let-7a induced cell cycle arrest, inhibited CCND2 and proliferation of human prostate cancer cells (*Homo sapiens*) [Dong, 2010].
- microRNA-497 down-regulated CCND2 and induced apoptosis via the Bcl-2/Bax-caspase 9- caspase 3 pathway in HUVECs (*Homo sapiens*) [Wu, 2016].
- micoRNA-26a regulated p53-mediated apoptosis and CCND2 and CCNE2 in mouse hepatocyte (*Mus musculus*) [Zhou, 2016].

Key Event Relationship Description

Cell cycle dysregulation leads to apoptosis. Cell cycles characterized by the DNA content changes regulate cell death and cell proliferation [Lynch, 1986].

Evidence Supporting this KER

microRNA-497, potentially targeting Bcl2 and Cyclin D2 (CCND2), induced apoptosis via the Bcl-2/Bax - caspase 9 - caspase 3 pathway and CCND2 protein in human umbilical vein endothelial cells (HUVECs) [Wu, 2016]. The microRNA-497 activated caspases 9 and 3, and decreased Bcl2 and CCND2 [Wu, 2016]. CCND2 is an important cell cycle gene that induces G_1 arrest [Li, 2012], and deregulated CCND2 is implicated in cell proliferation inhibition [Wu, 2016, Mermelstein, 2005, Dong, 2010].

Biological Plausibility

The incidence of apoptosis was increased in vincristine-treated cells, in which metaphases were arrested, compared to untreated cells, which indicates that cell cycle dysregulation leads to apoptosis [Sarraf, 1986]. Cell gain and loss are balanced with mitosis and apoptosis [Cree, 1987]. Apoptosis is mediated by caspase activation [Porter, 1999]. Caspase-3 is activated in the programmed cell death, and the pathways to caspase-3 activation include caspase-9 and mitochondrial cytochrome c release [Porter, 1999]. The activation of caspase-3 leads to apoptotic

chromatin condensation and DNA fragmentation [Porter, 1999]. Sinularin, a marine natural compound, exhibited DNA damage and induced G_2/M cell cycle arrest, followed by apoptosis in human hepatocellular carcinoma HepG2 cells [Chung, 2017]. Sinularin induced caspases 8, 9, and 3, and pro-apoptotic protein Bax, whereas it decrease the anti-apoptotic Bcl-2 protein expression level [Chung, 2017].

Empirical Evidence

- Cell cycle arrest such as G_1 arrest and G_1/S arrest are observed in apoptosis [Li, 2012, Dong, 2010].
- microRNA-1 and microRNA-206 represses CCND2, while microRNA-29 represses CCND2 and induces G_1 arrest and apoptosis in rhabdomyosarcoma [Li, 2012].
- The treatment with HDAC inhibitor, methoxyacetic acid (MAA) in prostate cancer cells induced growth arrest and apoptosis [Parajuli, 2014]. MAA blocks G_1/S transition of cell cycle [Parajuli, 2014]. MAA reduces CDK4 and CDK2, and decreases protein expression of BIRC2 and activates caspase 7 and 3 [Parajuli, 2014].

Uncertainties and Inconsistencies

MAA induces apoptosis, however, the MAA-induced changes of BCL2, BAX, BCL2L1, BAD, BID, MCL1, and CFLAR, pro-apoptotic and anti-apoptotic genes were not observed [Parajuli, 2014]. microRNA-497 induce activation of caspase-9 and -3, followed by apoptosis, however, the caspase-9 and -3 protein levels were repressed by the ectopic expression of microRNA-497, which remains uncertain [Wu, 2016].

Quantitative Understanding of the Linkage

Cell proliferation which was determined at daily intervals agter a 24-hr pulse of [3H]thymidine changed as the amount of DNA in the cultures changed. Cell death which was measured by lactic dehydrogenase (LDH) activity in the medium changed in parallel with the changes in cell proliferation [Lynch, 1986]. The decrease in total DNA was measured, the increase in cell death was observed [Lynch, 1986].

Time-scale

MAA (5 mM) decreases CDK4, CDK2 expression in 48 hrs after the treatment, which indicates the G_1 arrest [Parajuli, 2014]. MAA (5 mM) decreases the protein expression of procaspase 7 and 3 in 24 to 72 hrs after the treatment, indicating the activation of caspases 7 and 3 [Parajuli, 2014].

References

Lynch MP et al. (1986) Evidence for soluble factors regulating cell death and cell proliferation in primary cultures of rabbit endometrial cells grown on collagen. Proc Natl Acad Sci USA 83: 4784-4788

Wu R et al. (2016) microRNA-497 induces apoptosis and suppressed proliferation via the Bcl-2/Bax-caspase9-caspase 3 pathway and cyclin D2 protein in HUVECs. PLoS One 11: e0167052

Li L et al. (2012) Downregulation of microRNAs miR-1, -206 and -29 stabilizes PAX3 and CCND2 expression in rhabdomyosarcoma. Lab Invest 92: 571-583

Mermelshtein A et al. (2005) Expression of F-type cyclins in colon cancer and in cell lines from colon carcinomas. Br J Cancer 93: 338-345

Dong Q et al. (2010) microRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One 5: e10147

Kerr JFR et al. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257

Sarraf CE and Bowen ID (1986) Kinetic studies on a murine sarcoma and an analysis of apoptosis. Br J Cancer 54: 989-998

Cree IA et al. (1987) Cell death in granulomata: the role of apoptosis J Clin Pathol 40: 1314-1319

Porter AG and Janicke RU. (1999) Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6: 99-104

Chung TW et al. (2017) Sinularin induces DNA damage, G2/M phase arrest, and apoptosis in human hepatocellular carcinoma cells. BMC Complement Altern Med 17: 62

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2:300-313

Zhou J et al. (2016) miR-26a regulates mouse hepatocyte proliferation via directly targeting the 3' untranslated region of CCND2 and CCNE2. Hepatobiliary Pancreat Dis Int 15: 65-72

Relationship: 1735: Apoptosis leads to spermatocyte depletion (https://aopwiki.org/relationships/1735)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**adjacent**	**High**	**Not Specified**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High

The relationship between apoptosis and spermatocyte depletion is likely well conserved between species.

- Spermatogenesis was inhibited by knockdown of Sucla2, a β subunit of succinyl coenzyme A synthase, via apoptosis in the mouse spermatocyte (*Mus musculus*) [Huang, 2016].
- The suppression of microRNA-21 led to apoptosis of spermatogonial stem cell-enriched germ cell cultures and the decrease in the number of spermatogonial stem cells in mice (*Mus musculus*) [Niu Z, 2011].
- MAA induced apoptosis and depletion of spermatocytes in adult rats (*Rattus norvegicus*) [Brinkworth, 1995].
- The apoptosis and proliferation inhibition induced by MAA, a HDAC inhibitor, was measured in human prostate cancer cell lines (*Homo sapiens*) [Parajuli, 2014].
- The cell viability inhibition induced by SAHA or TSA , which are HDAC inhibitors, was observed in NHDFs (*Homo sapiens*) [Glaser, 2003].
- The proliferation of the $HDAC^{-/-}$ ES cells was inhibited compared to $HDAC^{+/+}$ ES cells (*Homo sapiens*) [Zupkovitz, 2010].
- It has been reported that mice lacking both *Ink4c* and *Ink4d* ,cyclin D-dependent kinase inhibitors, produced few mature sperm, and the residual spermatozoa had reduced motility and decreased viability (*Mus musculus*) [Zindy, 2001].
- The sperm counts in the cauda epidydimis of rats exposed to butylparaben were significantly decreased (*Rattus norvegicus*) [Oishi, 2001].
- MAA treatment induced spermatocyte death in Sprague-Dawley rats (*Rattus norvegicus*) [Wade, 2008].

Key Event Relationship Description

Apoptosis results in spermatocyte depletion via cell death. HDAC inhibitor, MAA, induced apoptosis and spermatocyte depletion at stages IX-II [Brinkworwth, 1995]. Induced apoptosis during development of germ cells results in progressive depletion of spermatocyte.

Evidence Supporting this KER

Biological Plausibility

In the mouse spermatocyte, spermatogenesis was inhibited by knockdown of Sucla2, a b subunit of succinyl coenzyme A synthase, via apoptosis [Huang, 2016]. The prolonged cryptorchidism leads to germs cell apoptosis and testicular sperm count decrease [Barqawi, 2004]. CD147 was reported to regulate apoptosis in mouse testis and spermatocyte cell line (GC-2 cells) via NFκB pathway [Wang, 2017].

Empirical Evidence

MicroRNA-21 regulates the spermatogonial stem cell homeostasis, in which suppression of microRNA-21 with anti-miR-21 oligonucleotides led to apoptosis of spermatogonial stem cell-enriched germ cell cultures and the decrease in the number of spermatogonial stem cells [Niu, 2011].

Uncertainties and Inconsistencies

The process of apoptosis is necessary for the meitosis of the stem cell differentiation in the testis, which remains in question for the regulation of spermatocyte deletion and testis atrophy/weight loss [Dym, 1994].

References

Brinkworth M et al. (1995) Identification of male germ cells undergoing apoptosis in adult rats. J Reprod Fertil 105: 25-33

Huang S et al. (2016) Knockdown of Sucla2 decreases the viability of mouse spermatocytes by inducing apoptosis through injury of the mitochondrial function of cells. Folia Histochem Cytobiol 54: 134-142

Barqawi A et al. (2004) Effect of prolonged cryptorchidism on germ cell apoptosis and testicular sperm count. Asian J Androl 6: 47-51.

Wang C et al. (2017) CD147 regulates extrinsic apoptosis in spermatocytes by modulating NFkB signaling pathways. Oncotarget 8: 3132-3143

Niu Z et al. (2011) microRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. Proc Natl Acad Sci 108: 12740-12745

Dym M. (1994) Spermatogonial stem cells of the testis. Proc Natl Acad Sci USA 91: 11287-11289

Bose R et al. (2017) Ubiquitin ligase Huwe1 modulates spermatogenesis by regulating spermatogonial differentiation and entry into meiosis. Sci Rep 7: 17759

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2: 300-313

Glaser KB et al. (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2:151-163

Zupkovitz G et al. (2010) The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation. Mol Cell Biol 30:1171-1181

Zindy F et al. (2001) Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors $p18^{Ink4c}$ and $p19^{Ink4d}$. Mol Cell Biol 21:3244-3255

Oishi S. (2001) Effects of butylparaben on the male reproductive system in rats. Toxicol Indust Health 17:31-39

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Relationship: 1734: spermatocyte depletion leads to testicular toxicity (https://aopwiki.org/relationships/1734)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**adjacent**	**High**	**Not Specified**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High

The relationship between spermatocyte depletion and testicular toxicity is likely well conserved between species.

- ME and MAA induced spermatocyte depletion and testicular atrophy in rat (*Rattus norvegicus*) [Beattie, 1984].
- Ethylene glycol monomethyl ether induced depletion of late spermatocytes and testicular atrophy in F344 rat (*Rattus norvegicus*) [Chapin, 1984].
- The epididymal tubules of rats with testicular degeneration had low sperm density (*Rattus norvegicus*) [Lee, 1993].
- Hydroxyurea induced spermatocyte reduction and testicular atrophy (*Mus musculus*) [Wiger, 1995].

Key Event Relationship Description

Spermatocyte depletion leads to testicular toxicity such as testicular atrophy with decrease in size. The spermatocyte depletion is involved in testicular atrophy and testicular toxicity [Chapin, 1984].

Evidence Supporting this KER

Biological Plausibility

Spermatocyte depletion caused by apoptosis leads to the testicular toxicity. Apoptosis is a basic biological phenomenon in which the cells are controlled in the atrophy of various tissues and organs through the deletion and turnover, as well as in tumor regression [Kerr, 1972].

Empirical Evidence

2-methoxyethanol (ME) or its major metabolite, methoxyacetic acid (MAA), HDAC inhibitor, induced spermatocyte depletion and testicular atrophy [Beattie, 1984].

Uncertainties and Inconsistencies

Spermatogonial stem cell self-renewal and spermatocyte meiosis are regulated by Sertoli cell signaling, which suggests us that various pathways in spermatocytes or spermatogonia are involved in the spermatocyte deletion and testis atrophy/weight loss [Chen, 2015].

References

Chapin RE et al. (1984) The effects of ethylene glycol monomethyl ether on testicular histology in F344 rats. J Andro 5: 369-380

Kerr JFR et al. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257

Beattie PJ, et al. (1984) The effect of 2-methoxyethanol and methoxyacetic acid on Sertoli cell lactate production and protein synthesis in vitro. Toxicol Appl Pharmacol 76: 56-61

Chen S and Liu Y. (2015) Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. Reproduction 149: R159-R167

Abedi N et al. (2017) Short and long term effects of different doses of paracetamol on sperm parameters and DNA integrity in mice. Middle East Fertility Society Journal 22: 323-328

Wiger R et al. (1995) Effects of acetaminophen and hydroxyurea on spermatogenesis and sperm chromatin structure in laboratory mice. Reprod Toxicol 9: 21-33

De Rooij DG et al. (2001) Proliferation and differentiation of spermatogonial stem cells. Reproduction 121: 347-354

De Rooij DG. (1998) Stem cells in the testis. Int J Exp Path 79: 67-80

Lee KP et al. (1993) Testicular degeneration and spermatid retention in young male rats. Toxicol Pathol 21: 292-302

List of Non Adjacent Key Event Relationships

Relationship: 1715: Histone deacetylase inhibition leads to Cell cycle, disrupted (https://aopwiki.org/relationships/1715)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**non-adjacent**	**High**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

MAA induced G_1 cell cycle arrest in human prostate cancer cells (*Homo sapiens*) [Parajuli, 2014]. Apicidin induced G_1 cell cycle arrest in HeLa cells (*Homo sapiens*) [Han, 2000].

Key Event Relationship Description

HDAC inhibition leads to cell cycle arrest including G_1/S phase arrest [Falkenberg, 2014]. The HDAC inhibition-induced cell cycle arrest is the mediated by transcriptional changes of the CDK inhibitors such as p21 [Falkenberg, 2014].

Evidence Supporting this KER

Biological Plausibility

The knockdown of HDACs may induce antitumor effects such as cell cycle arrest and inhibition of proliferation [Falkenberg, 2014]. In leukemia, acute myloid leukaemia 1-ETO, oncogenic fusion protein, recruits the variety of the proteins including HDACs to form multiprotein complexes to repress the cell cycle inhibitors, which suggests that the HDAC inhibition leads to cell cycle dysregulation [Falkenberg, 2014].

Empirical Evidence

- HDAC inhibition with SAHA, TSA and MS-27-275 induced acetylation of histone H4, up-regulation of cyclin-dependent kinase inhibitor p21, and inhibition of proliferation in human bladder carcinoma cells [Glaser, 2003].
- Apicidin [cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)], a fungal metabolite HDI, inhibits proliferation of tumor cells via p21 induction [Han, 2000]. Apicidin induced hyperacetylation of histone H4, up-regulation of p21, and G_0/G_1 cell cycle arrest in HeLa cells [Han, 2000].
- HDAC inhibition leads to cell cycle arrest, where G_1/S phase arrest occurs via up-regulation of p21 [Falkenberg, 2014].
- Loss of HDAC1 in mouse embryonic stem (ES) cells has demonstrated the acetylation of histones H3 and H4, up-regulation of cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ and inhibition of proliferation [Lagger, 2002].
- G_1/S transition blockade was observed in MAA-treated prostate cancer cells [Parajuli, 2014].

Uncertainties and Inconsistencies

MAA, a HDI, induced cell cycle arrest and up-regulation of p21 expression, and inhibited prostate cancer cell growth [Parajuli, 2014]. The involvement of p53/p63/p73 in up-regulation of p21 induced by HDAC inhibition is not fully elucidated, where time course of the p21 and p53/p63/p73 mRNA expression has demonstrated the cell-line specific differences in the responses in 4 human prostate cancer cell lines LNCaP, C4-2B, PC-3 and DU-145 [Parajuli, 2014].

Quantitative Understanding of the Linkage

MAA (20 mM) induced G_1 cell cycle arrest upon the treatment for 24 hrs in LNCaP, C4-2B, PC-3 and DU-145 human prostate cancer cell lines [Parajuli, 2014]. Almost 80% of the cells were arrested in G_1 phase upon stimulation of MAA, whereas approximately 40 to 60 % of the cells were in G_1 phase without MAA treatment [Parajuli, 2014].

Time-scale

MAA (5 mM) induced p21 up-regulation in 12 to 72 hrs in LNCaP, C4-2B, PC-3 and DU-145 human prostate cancer cell lines [Parajuli, 2014].

References

Falkenberg KJ and Johnstone RW. (2014) Histone deacetylases and their inhibitors in cancer, neurological disease and immune disorders. Nat Rev Drug Discov 13:673-691

Glaser KB et al. (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2:151-163

Han JW et al. (2000) Apicidin, a histone deacetylase inhibitor, inhibits proliferation of tumor cells via induction of $p21^{WAF1/Cip1}$ and gelsolin. Cancer Res 60:6068-6074

Lagger G et al. (2002) Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 21:2672-2681

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2:300-312

Relationship: 1716: Histone deacetylase inhibition leads to Apoptosis (https://aopwiki.org/relationships/1716)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**non-adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

AR-42 inhibited proliferation of human pancreatic cancer cells (*Homo sapiens*) [Henderson, 2016]. SAHA inhibited proliferation of NHDF (*Homo sapiens*) [Glaser, 2003]. MAA induced apoptosis in human prostate cancer cell lines (*Homo sapiens*) [Parajuli, 2014]. HDAC-deficient mouse ES cells showed decrease in proliferation (*Mus musculus*) [Lagger, 2002].

Key Event Relationship Description

HDAC inhibition leads to cell death through the apoptotic pathways [Falkenberg, 2014]. Intrinsic apoptosis pathway requires BH3-only proteins, and BCL-2 protein overexpression inhibits apoptosis [Falkenberg, 2014].

Evidence Supporting this KER

Biological Plausibility

HDAC inhibition in cancer results in apoptosis with the up-regulation of pro-apoptotic B cell lymphoma 2 (BCL-2) family genes and down-regulation of pro-survival BCL-2 genes [Falkenberg, 2014]. The antitumor effect of HDAC inhibition includes cell death and apoptosis [Falkenberg, 2014].

Empirical Evidence

- HDAC-deficient mouse embryonic stem (ES) cells showed reduced proliferation rates with up-regulation of cyclin-dependent kinase inhibitors p21 and p27 [Lagger, 2002]. HDAC-null embryoid bodies showed a reduced inner cell mass and reduced colony formation [Lagger, 2002].
- HDAC inhibition by suberoylanilide hydroxamic acid (SAHA) inhibited proliferation of normal human dermal fibroblasts (NHDF) [Glaser, 2003].
- MAA-induced spermatocyte death is associated with histone acetylation increase [Wade, 2008].
- The HDAC inhibition induced p21 up-regulation, histone acetylation increase, and apoptosis markers such as BAK overexpression and suppression of phosphorylated AKT [Henderson, 2016].
- The administration of methoxyacetic acid can cause apoptosis in the germ cells of adult male rats [Brinkworth, 1995].

Quantitative Understanding of the Linkage

MAA (5 mM) induced apoptosis in prostate cancer cell lines, LNCaP, C4-2B, PC-3 and DU-145, in which apoptotic nucleosomes were calculated as absorbance at 405 nm – absorbance at 490 nm [Parajuli, 2014].

Time-scale

MAA (5 mM) decreased protein expression of BIRC2 and activated caspases 7 and 3 within 72 hrs [Parajuli, 2014].

References

Falkenberg KJ and Johnstone RW. (2014) Histone deacetylases and their inhibitors in cancer, neurological disease and immune disorders. Nat Rev Drug Discov 13:673-691

Lagger G et al. (2002) Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 21:2672-2681

Glaser KB et al. (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2:151-163

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

Henderson SE et al. (2016) Suppression of tumor growth and muscle wasting in a transgenic mouse model of pancreatic cancer by the novel histone deacetylase inhibitor AR-42. Neoplasia 18:765-774

Brinkworth MH et al. (1995) Identification of male germ cells undergoing apoptosis in adult rats. J Reprod Fertil 105:25-33.

Parajuli KR et al. (2014) Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis. Am J Clin Exp Urol 2:300-312

Relationship: 1717: Histone deacetylase inhibition leads to testicular toxicity (https://aopwiki.org/relationships/1717)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular toxicity (https://aopwiki.org/aops/212)	**non-adjacent**	**Moderate**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High

The administration of di(2-ethylhexyl)-phthalate induced testis atrophy in rats (*Rattus norvegicus*) [Oishi, 1994]. The administration of butylparaben resulted in decrease in sperm counts in rats (*Rattus norvegicus*) [Oishi, 2001]. MAA induced spermatocyte apoptosis in human testes (*Homo sapiens*) [Li, 1996].

Key Event Relationship Description

HDAC inhibition induced testicular toxicity including testis atrophy such as the decrease in size [Miller, 1982]. HDAC inhibition in cell culture resulted in the testicular toxicity including germ cell apoptosis and cell morphology change [Li, 1996]. Valproic acid, a HDAC inhibitor, caused a reduced testicular weight in the offspring in rats [Kallen, 2004].

Evidence Supporting this KER

Biological Plausibility

The HDAC inhibition induced cell death in spermatocytes in both rat and human seminiferous tubules [Li, 1996]. The HDAC inhibitor treatment resulted in degeneration in spermatocytes in rat seminiferous tubules [Li, 1996]. The HDAC inhibition induced the germ cell apoptosis in human testicular tissues [Li, 1996].

Empirical Evidence

- HDAC inhibitor, methoxyacetic acid (MAA), (300 mg/kg) induced testicular toxicity measured with testis weight loss [Miller, 1982].
- MAA induced apoptosis and degeneration in spermatocytes in human testicular tissue and 25-day rat seminiferous tubule cultures [Li, 1996].
- MAA-induced spermatocyte death with an association of histone acetylation increase [Wade, 2008].
- Doxorubicin, which has a testicular toxicity, induced caspase 3 activation and g-H2AX induction, apoptosis markers, in human lung cancer A549 cells [El-Awady, 2015, Yamazoe, 2015].
- Doxorubicin-resistant A549 cells showed reduced expression of HDAC1, 3 and 4 compared to A549 cells [El-Awady, 2015].
- MAA-induced apoptosis in male germ cells was modulated by Sertoli cells via P/Q type voltage-operated calcium channels [Barone, 2005].
- The *p.o.* administration of ethylene glycol monomethyl (500 mg/kg/day) in rats induced the testis or liver organ weight loss on 2, 4, 7 and 11 days or 24 hrs and 2, 4 and 7 days after treatment, respectively [Foster, 1983].
- The investigation of 2-methoxyethanol (2-ME)-induced testicular toxicity has revealed that the conversion of 2-ME to MAA is required in 2-ME-induced testicular toxicity [Moss, 1985].

- The exposure of MAA induced morphological changes on embryonic forelimbs [Dayan, 2014].

Uncertainties and Inconsistencies

Methyl and ethyl esters of *p*-hydroxybenzoic acid did not show spermatotoxic effects in rats (*Rattus norvegicus*) [Oishi, 2004]. It is reported that HDAC inhibition leads to teratogenic toxicity, whereas the correlation with testicular toxicity and teratogenic toxicity by HDAC inhibition is not fully understood [Menegola, 2006]. The oral administration of vorinostat (SAHA), a HDAC inhibitor, in Sprague-Dawley rats showed no indication of reproductive toxicity in drug-treated male rats, which suggested the involvement of some compensation mechanisms or digestion [Wise, 2008].

Quantitative Understanding of the Linkage

MAA administration (592 mg/kg/day) for 4 days showed testis weight loss in which the relative organ weights were 0.773 ± 0.022 g/100 g body weight, compared to 0.985 ± 0.028 g/100g body weight in control treated with water [Foster, 1984].

Time-scale

The relative testicular weight was decreased at day 2 after the treatment of 500 mg/kg/day treatment of ethylene glycol monomethyl ether [Foster, 1984]. The treatment of 5 mM MAA for 5 hrs induced the pachytene spermatocyte death in early stage tubules in 19 hrs [Li, 1996]. The degeneration in late spermatocytes was observed in late-stage tubules in 19 hrs after 5 mM MAA treatment for 5 hrs [Li, 1996].

References

Miller RR et al. (1982) Toxicity of methoxyacetic acid in rats. Fundam Appl Toxicol 2: 158-160

Li LH et al. (1996) 2-Methoxyacetic acid (MAA)-induced spermatocyte apoptosis in human and rat testes: an in vitro comparison. J Androl 17: 538-549

Kallen B (2004) Valproic acid is known to cause hypospadias in man but does not reduce anogenital distance or causes hypospadias in rats. Basic Clin Pharmacol Toxicol 94: 51-54

Wade MG et al. (2008) Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822-831

El-Awady RA et al. (2015) Epigenetics and miRNA as predictive markers and targets for lung cancer chemotherapy. Cancer Biol Ther 16: 1056-1070

Yamazoe Y. et al. (2015) Embryo- and testicular-toxicities of methoxyacetate and the related: a review on possible roles of one-carbon transfer and histone modification. Food Safety 3:92-107

Barone F. et al. (2005) Modulation of MAA-induced apoptosis in male germ cells: role of Sertoli cell P/Q-type calcium channels. Reprod Biol Endocrinol 3:13

Foster PM et al. (1983) Testicular toxicity of ethylene glycol monomethyl and monoethyl ethers in the rat. Toxicol Appl Pharmacol 69:385-399

Moss EJ et al. (1985) The role of metabolism in 2-methoxyethanol-induced testicular toxicity. Toxicol Appl Pharmacol 79:480-489

Dayan C and Hales BF. (2014) Effects of ethylene glycol monomethyl ether and its metabolite, 2-methoxyacetic acid, on organogenesis stage mouse limbs in vitro. Birth Defects Res (Part B) 101:254-261

Oishi S. (2004) Lack of spermatotoxic effects of methyl and ethyl esters of p-hydroxybenzoic acid in rats. Food Chem Tox 42: 1845-1849

Menegola E et al. (2006) Inhibition of histone deacetylase as a new mechanism of teratogensis. Birth Defects Res 78: 345-353

Wise LD et al. (2008) Assessment of female and male fertileity in Sprague-Dawley rats administered vorinostat, a histone deacetylase inhibitor. Birth Defects Res B Dev Reprod Toxicol 83: 19-26

Foster PM et al. (1984) Testicular toxicity produced by ethylene glycol monomethyl and monoethyl esters in the rat. Environ Health Perspect 57: 207-217

Oishi S. (1994) Prevention of Di(2-ethylhexyl)phthalate-induced testicular atrophy in rats by co-administration of the vitamin B12 derivative denosylcobalamin. Arch Environ Contam Toxicol 26: 497-503

Oishi S. (2001) Effects of butylparaben on the male reproductive system in rats. Tox Industr Health 17: 31-39