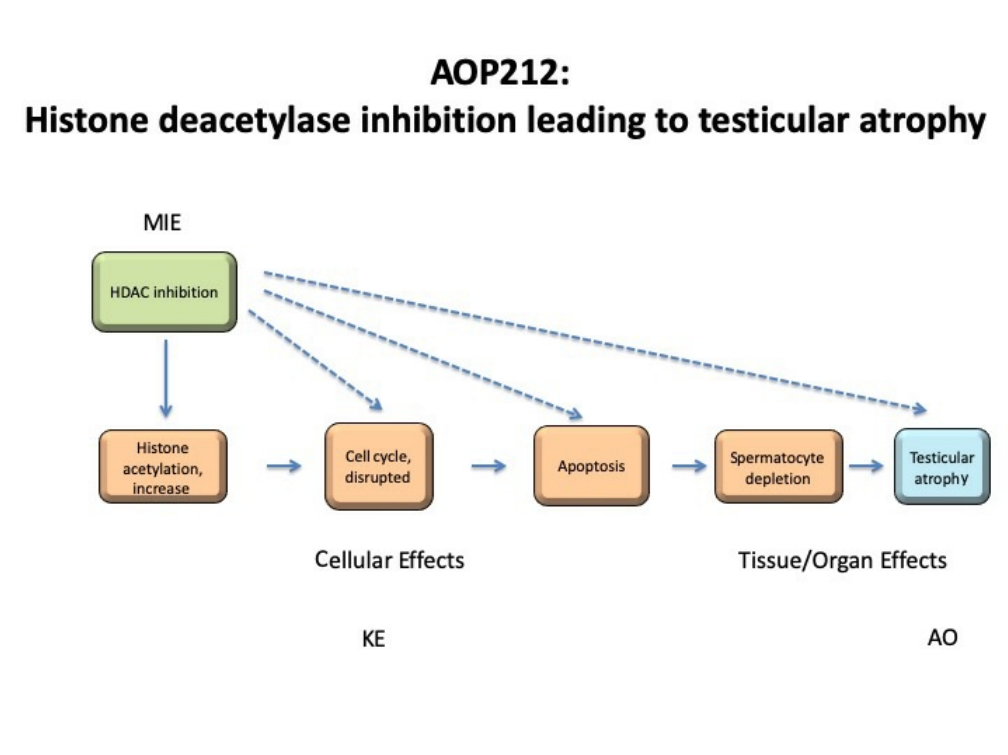
AOP ID and Title:

AOP 212: Histone deacetylase inhibition leading to testicular atrophy
Short Title: Histone deacetylase inhibition leading to testicular atrophy

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	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 been fully elucidated. Histone deacetylase inhibitors (HDIs) are approved as anti-cancer drugs since HDIs have apoptotic effects in cancer cells. HDIs include short-chain fatty acids, hydroxamic acids, benzamides, and epoxides. The intracellular mechanisms of induction of the spermatocyte apoptosis by HDIs are suggested as histone deacetylase (HDAC) inhibition as MIE, histone acetylation increase, disrupted cell cycle, apoptosis, and spermatocyte depletion as KEs. The adverse outcome has been defined as testicular atrophy. The HDIs inhibit deacetylation of the histone, leading to an increase in histone acetylation. The apoptosis induced by the disrupted cell cycle leads to spermatocyte depletion and testis atrophy. This AOP may be one of the pathways induced by HDIs, which suggests the pathway networks of protein hyperacetylations.

[Abbreviation] AOP: adverse outcome pathway, HDAC: histone deacetylase, HDI: HDAC inhibitor, KE: key event, MIE: molecular initiating event, MAA: 2-Methoxyacetic acid, or Methoxyacetic acid

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	Histone deacetylase inhibition
2	KE	1503	Histone acetylation, increase	Histone acetylation, increase
3	KE	1505	Cell cycle, disrupted	Cell cycle, disrupted
4	KE	1262	Apoptosis	Apoptosis
5	KE	1515	Spermatocyte depletion	Spermatocyte depletion
6	AO	1506	Testicular atrophy	Testicular atrophy

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Histone deacetylase inhibition	adjacent	Histone acetylation, increase	High	Moderate
Histone acetylation, increase	adjacent	Cell cycle, disrupted	Moderate	Moderate
Cell cycle, disrupted	adjacent	Apoptosis	Moderate	Moderate
Apoptosis	adjacent	Spermatocyte depletion	High	Not Specified
Spermatocyte depletion	adjacent	Testicular atrophy	High	Not Specified
Histone deacetylase inhibition	non-adjacent	Cell cycle, disrupted	High	Moderate
Histone deacetylase inhibition	non-adjacent	Apoptosis	Moderate	Moderate
Histone deacetylase inhibition	non-adjacent	Spermatocyte depletion	Moderate	Moderate
Histone deacetylase inhibition	non-adjacent	Testicular atrophy	Moderate	Moderate

Stressors

Name	Evidence
Methoxyacetic acid	High
Butyrate	High
Trichostatin A	High
Valproate	Moderate
Rocilinostat / Ricolinostat	High

Rocilinostat / Ricolinostat

Rocilinostat / Ricolinostat is the first oral selective HDAC6 inhibitor.

Ricolinostat plus lenalidomide, and dexamethasone in relapsed or refractory multiple myeloma: a multicentre phase 1b trial

By: Yee, Andrew J.; Bensinger, William I.; Supko, Jeffrey G.; Voorhees, Peter M.; Berdeja, Jesus G.; Richardson, Paul G.; Libby, Edward N.; Wallace, Ellen E.; Birrer, Nicole E.; Burke, Jill N.; et al

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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. The activity of histone acetyltransferase (HAT) in testis nuclear protein was increased with MAA addition [Wade et al., 2008].

KE1 => KE2: Histone acetylation, increase leads to cell cycle, disrupted	Biological Plausibility of the KE1 => KE2 is moderate. Rationale: Gene transcription is regulated by histone acetylation [Struhl, 1998]. Acetylation of histones neutralizes the positive charge of the histones. Thus, less compacted DNA can be bound more easily by transcription factors and transcribed. In the models proposed for the relationship between histone acetylation and transcription, histone acetylation can be untargeted and occur at both promoter and non-promoter regions, targeted generally to promoter regions, or targeted to specific promoters by gene-specific activator proteins [Richon et al., 2000; Struhl, 1998].
KE2 => KE3: Cell cycle, disrupted leads to apoptosis	Biological Plausibility of the KE2 => KE3 is moderate. Rationale: Prolonged cell cycle arrest will lead to either senescence or apoptosis. Especially for fast-dividing and still differentiating cells, such an arrest will most certainly induce apoptosis as the normal cellular program cannot be followed.
KE3 => KE4: Apoptosis leads to spermatocyte depletion	Biological Plausibility of the KE3 => KE4 is moderate. Rationale: During development and in tissue homeostasis, apoptosis is needed to control organ size. If apoptosis is induced at a higher rate, one can assume it leading to atrophy of the target organ. Especially when target organ/target cells are fast replicating, abnormal levels of apoptosis will lead to depletion.
KE4 => AO: Spermatocyte depletion leads to testicular atrophy	Biological Plausibility of the KE4 => AO is moderate. Rationale: Spermatocyte depletion is one of the main characteristics of testicular atrophy.
2. Support for Essentiality of KEs	
KE2: Cell cycle, disrupted	The essentiality of the KE2 is moderate. The rationale for the Essentiality of KEs in the AOP: HDAC1-deficient embryonic stem cells showed reduced proliferation rates, which correlates with decreased cyclin-associated kinase activities and elevated levels of the cyclin-dependent kinase inhibitor 1A, a cell cycle regulator p21 [Lagger et al., 2002]. Loss of HDAC1 leads to significantly reduced overall deacetylase activity, hyperacetylation of a subset of histones H3 and H4 [Lagger et al., 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: HDAC inhibitors increase histone acetylation in the brain [Schroeder et al., 2013]. 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 [^{3}H]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].
KE1 => KE2: Histone acetylation, increase leads to	Empirical Support of the KE1 => KE2 is moderate. Rationale: Increase in histone acetylation by HDAC inhibition induces the cell cycle regulator expression and inhibits progression through the cell cycle. Histone acetylation regulates the gene transcriptional mechanism [Struhl, 1998]. Acetylation of histones promotes the RNA polymerase reaction [Allfrey et al., 1964; Pogo et al., 1966].

cell cycle, disrupted	Since several results supported a model in which increased histone acetylation is targeted to a specific gene and occurs throughout the entire genome, not just the promoter regions, histone acetylation may lead to gene transcription of the cell cycle regulator [Richon et al., 2000].
KE2 => KE3: Cell cycle, disrupted leads to apoptosis	Empirical Support of the KE2 => KE3 is moderate. Rationale: Cell cycle arrests such as G_1 arrest and G_1/S arrest are observed in apoptosis [Li et al., 2012; Dong et al., 2010]. microRNA-1 and microRNA-206 repress CCND2, while microRNA-29 represses CCND2 and induces G_1 arrest and apoptosis in rhabdomyosarcoma [Li et al., 2012].
KE3 => KE4: Apoptosis leads to spermatocyte depletion	Empirical Support of the KE3 => KE4 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 et al., 2011].
KE4 => AO: Spermatocyte depletion leads to testicular atrophy	Empirical Support of the KE4 => AO is high. Rationale: The testicular atrophy seen in 2-methoxyethanol (2-ME), or its major metabolite MAA, treated rats *in vivo* and in human, and rat *in vitro* culture was associated with spermatocyte depletion [Beattie et al., 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
mouse	Mus musculus	Moderate	NCBI
rat	Rattus norvegicus	High	NCBI

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 et al., 1982]. HDAC inhibition in cell culture resulted in testicular toxicity including germ cell apoptosis and cell morphology change [Li et al., 1996].
KE1: Histone acetylation, increase	The HDAC inhibition induced cell death in spermatocytes in both rat and human seminiferous tubules [Li et al., 1996].
KE2: Cell cycle, disrupted	In HDAC1-/- fibroblast lines, an increase in the number of cells in the G_1 phase and a decrease in the number of cells in the S phase were observed, which indicates the importance of HDAC inhibition in cell cycle regulation [Zupkovitz et al., 2010].
KE3: Apoptosis	HDAC inhibition leads to cell death through the apoptotic pathways [Falkenberg et al., 2014].
	The HDAC inhibition induced cell death in spermatocytes in both rat and human

KE4: Spermatocyte depletion	seminiferous tubules [Li et al., 1996]. The HDAC inhibitor treatment resulted in degeneration in spermatocytes in rat seminiferous tubules [Li et al., 1996]. The HDAC inhibition induced germ cell apoptosis in human testicular tissues [Li et al., 1996].

Weight of Evidence Summary

Biological plausibility, coherence, and consistency of the experimental evidence

The available data supporting the AOP are logical, 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), an HDI, in mice resulted in male meiosis impairment showed the involvement of p53-noxa-caspase-3 apoptotic pathway in TSA-induced spermatocyte apoptosis [Fenic et al., 2008]. Another study showed that MAA-induced up-regulation of p21 expression is mediated through histone hyperacetylation and independent of p53/p63/p73 [Parajuli et al., 2014].

- NF-kappaB pathway

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

- Communication with Sertoli cells

The present AOP focuses on testicular atrophy by HDAC inhibition-induced apoptosis in spermatocytes, however, the signaling in Sertoli 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 and Liu, 2015].

- Decrease in deoxynucleotide pool by MAA

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

- Spermatocyte depletion by necrosis

Spermatocyte may be decreased by necrosis. Cell death mechanisms other than apoptosis, such as necrosis, may be considered for spermatocyte depletion.

Quantitative Consideration

Concordance of dose-response relationships

This is a quantitative description of 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 evidence shows

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 et al., 1983; Miller et al., 1982].

Considerations for Potential Applications of the AOP (optional)

The AOP may be useful in the risk assessment on several types of HDI molecules including anti-cancer drugs, as well as other types of chemicals, biocides, or pesticides. 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 to testicular atrophy may provide important insights into the 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/ricolinostat are clinically evaluated as anti-cancer drugs in clinical trials [Yee et al., 2016]. The AOP may be useful for the risk assessment of chemicals, since possible applications of HDAC inhibitors include the enhancement of salinity tolerance to increase agricultural sustainability. Other potential applications of the AOP include the risk assessment of biocides or pesticides, considering that HDAC inhibitors are being investigated as insecticides or amoebicides [Bagnall et al., 2017; Lee et al., 2020].

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Appendix 1

List of MIEs in this AOP

Event: 1502: Histone deacetylase inhibition

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 atrophy	MolecularInitiatingEvent
Aop:274 - Histone deacetylase inhibition leads to impeded craniofacial development	MolecularInitiatingEvent
Aop:275 - Histone deacetylase inhibition leads to neural tube defects	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 et al., 2003; Ropero and Esteller, 2007; Villar-Garea et al., 2004]. There is a report showing that TSA and butyrate competitively inhibit HDAC activity [Sekhavat et al., 2007]. HDIs inhibit preferentially HDACs with some selectiveness [Hu et al., 2003]. TSA (Trichostatin A) inhibits class I and II of HDACs, while butyrate inhibits class I and IIa (HDACs 4, 5, 7, 9) of HDACs [Ooi et al., 2015; Park and Sohrabji, 2016; Wagner et al., 2015]. TSA inhibits HDAC1, 2, and 3 [Damaskos et al., 2016], whereas MS-27-275 has an inhibitory effect for HDAC1 and HDAC3 (IC_{50} value of ~0.3 microM and ~8 microM, respectively), but no effect for HDAC8 (IC_{50} value >100 microM) [Hu et al., 2003].

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

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

- HDAC inhibition restores the rate of resorption of subretinal blebs in hyperglycemia in brown Norway rat and HDAC activity was inhibited with HDIs in human ARPE19 cells [Desjardins et al., 2016].
- Treatment of HDIs inducing HDAC inhibition showed anti-tumor effects in human non-small cell lung cancer cells [Ansari et al., 2016; Miyanaga et al., 2008].
- HDAC acetylation level was increased by HDIs in the MRL-lpr/lpr murine model of lupus splenocytes [Mishra et al., 2003].

- SAHA increased histone acetylation in the brain and spleen of mice [Hockly et al., 2003].
- MAA inhibits HDAC activity in HeLa cells and spleens from C57BL/6 mice [Jansen et al., 2004].
- It is also reported that MAA inhibits HDAC activity in testis cytosolic and nuclear extract of juvenile rats (27 days old) [Wade et al., 2008].
- VPA and TSA inhibit HDAC enzymatic activity in the mouse embryo and human HeLa cell nuclear extract [Di Renzo et al., 2007].
- The treatment with HDAC inhibitors, phenylbutyrate (PB) (2 mM) and TSA (200 nM), inhibits HDAC in adjuvant arthritis synovial cells derived from rats, causing higher acetylated histone [Chung et al., 2003].

Key Event Description

Nucleosomes consist of eight core histones, two of each histone H2A, H2B, H3, and H4 [Damaskos et al., 2017]. DNA strands (about 200 bp) wind around the core histones, which can be modified on their N-terminal ends. One possible modification is the acetylation of lysine residues, which decreases the binding strength between DNA and the core histone. Histone deacetylases (HDACs) hydrolyze the acetyl residues [Damaskos et al., 2017]. HDACs remove the acetyl groups from the lysine residues leading to the formation of a condensed and transcriptionally silenced chromatin. Thus, the inhibition of HDAC blocks this action and can result in hyperacetylation of histones associated mostly with increases in transcriptional activation. Histone deacetylase inhibitor (HDI) inhibits HDAC, causing increased acetylation of the histones and thereby facilitating binding of transcription factors [Taunton et al., 1996].

It is known that eukaryotic HDAC isoforms are classified into four classes: class I HDACs (isoforms 1, 2, 3, 8), class II HDACs (isoforms 4, 5, 6, 7, 9, 10), class III HDACs (the sirtuins), and HDAC11 [Gregoretti et al., 2004; Weichert, 2009; Barneda-Zahonero and Parra, 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 be expressed 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 and Parra, 2012]. SirT1 is highly expressed in testis, thymus, and multiple types of germ cells [Bell et al., 2014]. HDAC11 expression is enriched in the kidney, brain, testis, heart, and skeletal muscle [Barneda-Zahonero and Parra, 2012]. The members of classes 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 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 in 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 exerts 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. Some *in silico* methods including molecular modeling, virtual screening, and molecular dynamics are used to find the common HDAC inhibitor structures [Huang et al., 2016; Yanuar et al. 2016].

How it is Measured or Detected

The measurement of HDAC inhibition monitors changes in histone acetylation. HDAC inhibition can be detected directly by the measurement of HDAC activity using commercially available colorimetric or fluorimetric kits or indirectly by the increase of histone acetylation as the detection of global histone acetylation changes by Western blot or mass spectrometry (MS)-based proteomics methods or as detection of site-specific histone acetylation changes using chromatin immunoprecipitation (ChIP) or ChIP-on-Chip. The measurement methods include the immunological detection of histone acetylation with anti-acetylated histone antibodies [Richon et al., 2004]. The histones are isolated from pellets of cells treated with HDIs, followed by acid-urea-triton gel electrophoresis, western blotting, and immunohistochemistry [Richon et al., 2003]. 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 et al., 2016].

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List of Key Events in the AOP

Event: 1503: Histone acetylation, increase

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 atrophy	KeyEvent
Aop:275 - Histone deacetylase inhibition leads to neural tube defects	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 organisms to mammals.

MAA, an HDAC inhibitor, induces acetylation of histones H3 and H4 in Sprague-Dawley (*Rattus norvegicus)* [Wade et al., 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 et al., 2014].

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

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

Key Event Description

Gene transcription is regulated with the balance between acetylation and deacetylation. A dynamic balance of histone acetylation and histone deacetylation is typically catalyzed by enzymes with histone acetyltransferase (HAT) and HDAC activities. Histone acetylation relaxes chromatin and makes it accessible to transcription factors, whereas deacetylation is associated with gene silencing. The balance can be disturbed also by targeting HAT, not only HDACs. At least theoretically, an activation of HAT might lead to an increase in histone acetylation. The acetylation and deacetylation are modulated on the NH_3^+ groups of lysine amino acid residues in histones. HDAC inhibition promotes hyperacetylation by inhibiting the deacetylation of histones with classes of H2A, H2B, H3, and H4 in nucleosomes. [Wade et al., 2008]. The inhibition of HDACs results 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 et al., 2004]. Histone acetylation on chromatin can be measured using the labeling method with sodium [^{3}H]acetate [Gunjan et al., 2001]. The histone acetylation increase is detected as global histone acetylation changes by Western blot or mass spectrometry (MS)-based proteomics methods or as site-specific histone acetylation changes using chromatin immunoprecipitation (ChIP) or ChIP-on-Chip.

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Event: 1505: Cell cycle, disrupted

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 atrophy	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
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

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

Key Event Description

The disruption of the cell cycle leads to a decrease in cell number. The cell cycle consists of G_1, S, G_2, M, and G_0 phases. The cell cycle regulation is disrupted by the cell cycle arrest in certain cell cycle phases. The histone gene expression is regulated in cell cycle phases [Heintz et al., 1983].

How it is Measured or Detected

The percentage of cells at G_1, G_0, S, and G_2/M phases can be detected by flow cytometry [Li et al., 2013]. Cell cycle distribution was analyzed by fluorescence-activated cell sorter (FACS) analysis with a Partec PAS-II sorter [Zupkovitz et al., 2010]. The four cell-cycle phases in living cells can be measured with four-color fluorescent proteins using live-cell imaging [Bajar et al., 2016]. The incorporation of [^{3}H]deoxycytidine or [^{3}H]thymidine into cell DNA during the S phase can be monitored as DNA synthesis [Heintz et al., 1982].

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Event: 1262: Apoptosis

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	AdverseOutcome
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:212 - Histone deacetylase inhibition leading to testicular atrophy	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury	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
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Caenorhabditis elegans	Caenorhabditis elegans	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence

Sex	Evidence
Unspecific	High

Apoptosis is induced in human prostate cancer cell lines (*Homo sapiens*) [Parajuli et al., 2014].

Apoptosis occurs in B6C3F1 mouse (*Mus musculus*) [Elmore, 2007].

Apoptosis occurs in Sprague-Dawley rat (*Rattus norvegicus*) [Elmore, 2007].

Apoptosis occurs in the nematode (*Caenorhabditis elegans*) [Elmore, 2007].

Key Event Description

Apoptosis, the process of programmed cell death, is characterized by distinct morphology with DNA fragmentation and energy dependency [Elmore, 2007]. Apoptosis, also called “physiological cell death”, is involved in cell turnover, physiological involution, and atrophy of various tissues and organs [Kerr et al., 1972]. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances [Kerr et al., 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 et al., 2000]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation of the NHDF cells [Glaser et al., 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 et al., 2003]. The impaired proliferation was observed in HDAC1$^{-/-}$ ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz et al., 2010]. The present AOP focuses on the p21 pathway leading to apoptosis, however, alternative pathways such as NF-kB signaling pathways may be involved in the apoptosis of spermatocytes [Wang et al., 2017].

How it is Measured or Detected

Apoptosis is characterized by many morphological and biochemical changes such as homogenous condensation of chromatin to one side or the periphery of the nuclei, membrane blebbing and formation of apoptotic bodies with fragmented nuclei, DNA fragmentation, enzymatic activation of pro-caspases, or phosphatidylserine translocation that can be measured using electron and cytochemical optical microscopy, proteomic and genomic methods, and spectroscopic techniques [Archana et al., 2013; Martinez et al., 2010; Taatjes et al., 2008; Yasuhara et al., 2003].

DNA fragmentation can be quantified with comet assay using electrophoresis, where the tail length, head size, tail intensity, and head intensity of the comet are measured [Yasuhara et al., 2003].

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

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

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

Cleavage of PARP is detected with Western blotting [Parajuli et al., 2014].

Caspase-3 and caspase-9 activity is measured with the enzyme-catalyzed release of p-nitroanilide (pNA) and quantified at 405 nm [Wu et al., 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 et al., 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 and Groscurth, 1994].

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

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Event: 1515: Spermatocyte depletion

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 atrophy	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
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Male	High

There are pieces of evidence of spermatocyte depletion in different species.

Mature sperm counts were decreased and the residual spermatozoa had reduced motility and decreased viability (*Mus musculus)* [Zindy et al., 2001].

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

Spermatocyte death can be induced in Sprague-Dawley rats (*Rattus norvegicus*) [Wade et al., 2008].

Key Event Description

Spermatocytes are differentiated from spermatogonial stem cells via random proliferation, differentiation, and synchronized mitoses with several stages [Rooij, 2001]. In each step of the spermatogonial differentiation, different molecular mechanisms are activated in the testis [Rooij, 2001; de Kretser et al., 2016]. The stem cell factor (SCF) genes are involved in differentiation into A1 spermatogonia. The expression of cyclin D2 is regulated in the epithelial stage VIII when the aligned spermatogonia differentiate into A1 spermatogonia [Rooij, 2001]. Upon the apoptosis of spermatogonia, overexpression of the apoptosis-inhibiting proteins Bcl-2 and Bcl-xL and deficiency of the apoptosis-inducing protein Bax have been shown to cause an accumulation of spermatogonia in the testis, leading to apoptosis of all cells [Rooij, 2001].

How it is Measured or Detected

Traditional spermatocytes assessment includes sperm count and concentration (haemocytometer, automated image-based system), morphology and motility (microscope, automated image-based system) and viability (for example propidium iodide staining of necrotic cells, TUNEL assay staining apoptotic cells). In addition, there are functional tests such as assays for genetic integrity (e.g. via measurement of DNA fragmentation/integrity -Halosperm kit or reactive oxygen species) and fertilization defects (through various measures of sperm-zona pellucida (ZP) interaction, such as measurement of ZP-receptor binding).

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 a haematocytometer [Zindy et al., 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 testis cell analysis, fresh testes were dispersed using two-stage enzymatic digestion and incubated in BSA containing collagenase and DNase I [Wade et al., 2006]. The seminiferous tubules were further digested and cells were fixed in ice-cold 70% ethanol [Wade et al., 2006]. Relative proportions of spermatogenic cell populations were assessed in fixed cells using a flow cytometeric method [Wade et al., 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 et al., 2006].

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List of Adverse Outcomes in this AOP

Event: 1506: Testicular atrophy

Short Name: Testicular atrophy

Key Event Component

Process	Object	Action
Testicular atrophy	Testis	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular atrophy	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
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Male	High

- The decrease in testis weight associated with testicular cell damage was induced by ethylene glycol monomethyl ether (EGME) or MAA treatment in rats (*Rattus norvegicus*) [Foster et al., 1983].
- The number of spermatocytes, principally pachytene cells, is decreased by EGME treatment in CD-1 mice (*Mus musculus*) and CD rats (*Rattus norvegicus*) [Anderson et al., 1987].
- The testicular lesions induced by 2-methoxyethanol (or EGME) were observed in rats (*Rattus norvegicus*) and guinea pigs (*Cavia porcellus*), which are different in onset, characteristics, and severity [Ku et al., 1984].
- Spermatogenesis was disrupted by EGME treatment in rabbits (*Oryctolagus cuniculus*) [Foote et al., 1995].
- Testicular toxicity such as spermatocyte death in seminiferous tubule stages I-IV and stages XII-XIV was induced by dimethoxyhexane (DMH) treatment in Sprague-Dawley rats (*Rattus norvegicus*) [Wade et al., 2006].

Key Event Description

It is hypothesized that the testicular effects of 1,6-dimethoxyhexane (DMH) are caused by its metabolism to methoxyacetic acid (MAA) [Wade et al., 2006; Poon et al., 2004]. MAA produces testicular and thymic atrophy such as the decrease in size [Miller et al., 1982; Moss et al., 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

- Testicular atrophy can be assessed by testicular volume measurement using an orchidometer, rulers, calipers, and ultrasonography or by testis weighing and histopathologic examination.
- The testis weight is measured to detect testicular atrophy [Foster et al., 1983].
- The urinary zinc excretion and testicular zinc content are examined since zinc concentration has been shown to play an important role in the production of testicular injury [Foster et al., 1983].
- The testicular tissue structure is observed whether there are normal germinal epithelial cells and Leydig cells [Mercantepe et al., 2018]. The testis is fixed for observations by light microscopy or transmission electron microscopy [McDowell and Trump, 1976; Mercantepe et al., 2018].
- Changes in sperm are measured by computer-assisted sperm analysis [Foote et al., 1995].
- For the assessment of sperm morphology, eosin-stained sperm collected from the cauda epididymis is observed. 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) [Wade et al., 2006].
- For the measurement of the total number of condensed spermatids per testis, a weighed portion of the parenchyma from the left testis was homogenized [Wade et al., 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 et al., 2006].
- For the determination of total LDH and LDH-X in the supernatant of the homogenized testis fragment, enzyme activity was measured by monitoring the extinction of NAD absorbance [Wade et al., 2006].

Regulatory Significance of the AO

The testicular atrophy assessment is important for assessing the side effects of the medicines such as anti-cancer drugs, as well as the hazard and risk of chemicals. The testicular atrophy including a decrease in testis weight and sperm count, fertility, decrease in morphology and function of the sperm, can become one of the main endpoints as the adverse effects of the therapeutics. The unexpected effects of the therapeutics may be predicted with this Adverse Outcome (AO). In terms of chemical risk assessment, the AO may be related to the health effects caused by the usage of pesticides or biocides.

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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

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	adjacent	High	Moderate
Histone deacetylase inhibition leads to neural tube defects	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Oryctolagus cuniculus	Oryctolagus cuniculus	Moderate	NCBI
Brassica napus	Brassica napus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between HDAC inhibition and increase in histone acetylation is conceivably well conserved among various species including mammals.

- Hyperacetylation by HDIs such as SAHA and Cpd-60 are observed in mice (*Mus musculus*) [Schroeder et al., 2013].
- TSA induces acetylation of histone H4 in a time-dependent manner in mouse cell lines (*Mus musculus*) [Alberts et al., 1998].
- AR-42, a novel HDI, induces hyperacetylation in human pancreatic cancer cells (*Homo sapiens*) [Henderson et al., 2016].
- SAHA and MS-275 lead to the hyperacetylation of lysine residues of histones in human cell lines of epithelial (A549) and lymphoid origin (Jurkat) (*Homo sapiens*) [Choudhary et al., 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 et al., 2016].
- TSA induces the acetylation of histones H3 and H4 in *Brassica napus* microspore cultures (*Brassica napu*) [Li et al., 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 and Johnstone, 2014].

Histone acetylation is one of the major epigenetic mechanisms and belongs to the posttranslational modifications of histones. Histone acetyltransferase is setting the mark, and deacetylase (HDAC) is responsible for removing the acetyl group from specific lysine 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 the epigenetic regulation of gene transcription. Upon the inhibition of HDAC by HDIs, lysine in

histone remains acetylated which leads to transcriptional activation or repression, changes in DNA replication, and DNA damage repair [Wade et al., 2008].

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 several mechanisms are involved, including the posttranslational modifications of histones (reviewed in [Bannister and Kouzarides, 2011; Kouzarides, 2007]. For a long time, it is known that histones get acetylated and that this reaction is catalyzed by histone acetyltransferases (HAT) whereas the acetyl groups are removed by histone deacetylases (HDAC). Inhibition of HDACs by small-molecule compounds leads to hyperacetylation of the histones as the homeostasis of acetylation and deacetylation is disturbed (reviewed in [Gallinari et al., 2007]).

Empirical Evidence

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].

There exist several pieces of evidence showing the link between histone deacetylase inhibition and increase in histone acetylation as follows:

- 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].
- HDAC inhibition by HDIs leads to hyperacetylation of histone and a large number of cellular proteins such as NF-kappaB [Falkenberg and Johnstone, 2014; Chen et al., 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 et al., 2006].
- HDIs increase histone acetylation in the brain [Schroeder et al., 2013].
- More acetylation sites on the histones H3 and H4 are responsive to SAHA than to MS-275 indicating that an HDI selectivity exists [Choudhary et al., 2009].
- HDI AR-42 induces acetylation of histone H3 in a dose-response manner in human pancreatic cancer cell lines [Henderson et al., 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 et al., 2008].
- HDAC inhibition induced by valproic acid (VPA) leads to histone hyperacetylation in mouse teratocarcinoma cell line F9 [Eikel et al., 2006].
- Hyperacetylation of histone H3 was observed in HDAC1-deficient ES cells [Lagger et al., 2002].
- The treatment of MAA induced histone acetylation in H3K9Ac and H4K12Ac, as well as p53K379Ac [Dayan and Hales, 2014].

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 additional biological effects. It is also noted that HDAC functions as the catalytic subunits of the large protein complex, which suggests that the inhibition of HDAC by HDIs affects the function of the large multiprotein complexes of HDAC [Falkenberg and Johnstone, 2014]. Related-analysis are usually 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 et al., 2009].

Response-response relationship

SAHA or MS-275 treatment leads to an increase in acetylation of specific lysine residues on histones more than two-fold [Choudhary et al., 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 et al., 2009].

TSA (100 ng/ml) treatment leads to accumulation of 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 et al., 1990].

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Relationship: 1997: Histone acetylation, increase leads to Cell cycle, disrupted

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between increased histone acetylation and cell cycle disruption is likely well conserved between species. The present KER focuses on the pathway of p21, a cell-cycle regulator, leading to apoptosis. The examples are only given for mammals:

- Chidamide induced histone acetylation and cell cycle arrest in RPMI8226 and U266 human myeloma cells (*Homo sapiens*) [Yuan et al., 2019].
- TSA and sodium butyrate induced cell cycle regulator p21 mRNA expression in HT-29 human colon carcinoma cells (*Homo sapiens*) [Wu et al., 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 et al., 2004].
- Scriptaid, an HDI, up-regulated p21 mRNA expression in mouse embryonic kidney cells (*Mus musculus*) [Chen et al., 2011].

Key Event Relationship Description

Upon histone acetylation increase, cell cycle regulation is disrupted, where acetylation in the promoter region of the coding genes has a close correlation [Gurvich et al., 2004]. Transient histone hyperacetylation was sufficient for the activation of molecules involving cell cycle regulation such as inducing p21 gene expression [Wu et al., 2001]. Histone hyperacetylating agents butyrate and TSA induced mRNA expression of cell cycle regulator gene [Archer et al., 1998]. SAHA induced the accumulation of acetylated histones in the chromatin of the gene regulating cell cycle [Richon et al., 2000].

Evidence Supporting this KER

Biological Plausibility

Histone deacetylase inhibitors induce histone hyperacetylation and the activation of downstream molecules leading to the cell cycle arrest, which suggests the close correlation between histone hyperacetylation and cell cycle arrest [Yuan et al., 2019]. The histone acetylation regulates the gene transcription through the promoter region of the coding gene, which may lead to the overexpression of cell cycle regulators [Richon et al., 2000; Struhl, 1998]. Histone deacetylase inhibition leads to acetylation of histone, inducing the expression of cyclin-dependent kinase inhibitors, followed by a cell-cycle arrest [Li and Seto, 2016].

Empirical Evidence

- MAA induced histone acetylation of H4 in prostate cancer cells including LNCaP, C4-2B, PC-3, and DU-145 parallel with cyclin-dependent kinase inhibitor p21, a cell cycle regulator, mRNA level increase [Parajuli et al., 2014].
- HDIs accumulated acetylation of histones and induced cell cycle regulator p21 protein and mRNA expression [Richon et al., 2000; Wu et al., 2001].

Uncertainties and Inconsistencies

The histone acetylation causes cell cycle disruption in several pathways, in which the specific molecule involvement remains uncertain.

Quantitative Understanding of the Linkage

Histone acetylation occurs in a dose-dependent manner with the treatment of chidamide for 48 hrs [Yuan et al., 2019]. The expression of proteins related to G_0/G_1 cell cycle arrest, p21, and phosphorylated p53 is increased in a dose-dependent manner [Yuan et al., 2019].

Response-response relationship

Dose-response of histone acetylation and expression of p21 and phosphorylated p53 showed that treatment with 0.5, 1, or 2 microM of chidamide for 48hrs induced histone acetylation in RPMI8226 myeloma cells, while 2, 4, or 8 microM of chidamide for 48 hrs induced histone acetylation in U266 myeloma cells [Yuan et al., 2019]. Chidamide treatment in 0.5, 1, or 2 microM in RPMI8226

or 2, 4, or 8 microM in U266 induced G_0/G_1 arrest in the myeloma cells [Yuan et al., 2019]. 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 et al., 2004]. The p21 protein level was induced with the treatment of 0.25-2 mM of VPA in U937 cells [Gurvich et al., 2004].

Time-scale

Time course for histone H4 hyperacetylation in response to repeated doses of TSA every 8 hrs showed that histone hyperacetylation was peaked in 12 hrs in an 8-fold increase and showed a 5-fold increase in 24 hrs compared to control [Wu et al., 2001]. TSA (0.3 microM) induced cell cycle regulator p21 mRNA expression in 1 hr after stimulation and the induction is returned to the basal level in 24 hrs [Wu et al., 2001]. Sodium butyrate (5 mM) and repetitive doses of TSA (0.3 microM, every 8 hrs) induced the p21 mRNA level in 24 hrs in HT-29 cells [Wu et al., 2001]. Acetylation of p21 promoter and p21 mRNA induction were correlated in the treatment of valproic acid and analogs [Gurvich et al., 2004]. MAA-induced acetylation increases 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 et al., 2008].

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Relationship: 1712: Cell cycle, disrupted leads to Apoptosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Oryctolagus cuniculus	Oryctolagus cuniculus	Moderate	NCBI

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. The examples are only given for mammals:

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

Key Event Relationship Description

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

Evidence Supporting this KER

The microRNA-497, potentially targeting Bcl-2 and cyclin D2 (CCND2), activated caspases 9/3, and induced apoptosis via the Bcl-2/Bax - caspase 9 - caspase 3 pathway and CCND2 protein in human umbilical vein endothelial cells (HUVECs) [Wu, 2016]. CCND2 is an important cell cycle gene, of which a decrease in expression induces G_1 arrest [Li et al., 2012], and dysregulated CCND2 is implicated in cell proliferation inhibition [Wu et al., 2016; Mermelstein et al., 2005; Dong et al., 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 and Bowen, 1986]. Cell gain and loss are balanced with mitosis and apoptosis [Cree et al., 1987]. Apoptosis is mediated by caspase activation [Porter and Janicke, 1999]. Caspase-3 is activated in programmed cell death, and the pathways to caspase-3 activation include caspase-9 and mitochondrial cytochrome c release [Porter and Janicke, 1999]. The activation of caspase-3 leads to apoptotic chromatin condensation and DNA fragmentation [Porter and Janicke, 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 et al., 2017]. Sinularin induced caspases 8, 9, and 3, and pro-apoptotic protein Bax, whereas it decreases the anti-apoptotic Bcl-2 protein expression level [Chung et al., 2017].

Empirical Evidence

- Cell cycle arrests such as G_1 arrest and G_1/S arrest are observed in apoptosis [Li et al., 2012; Dong et al., 2010].
- microRNA-1 and microRNA-206 repress CCND2, while microRNA-29 represses CCND2 and induces G_1 arrest and apoptosis in rhabdomyosarcoma [Li et al., 2012].
- The blockade of G_1/S transition of cell cycle and reduction of CDK4 and CDK2, and apoptosis have occurred in HDAC inhibitor treatment [Parajuli et al., 2014].

Uncertainties and Inconsistencies

MAA induces CDK4 and CDK2 decreases, cell cycle arrest, and apoptosis, which may be regulated by several pathways [Parajuli et al., 2014].

Quantitative Understanding of the Linkage

Cell proliferation which was determined at daily intervals after a 24-hr pulse of [^{3}H]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 et al., 1986].

Response-response relationship

Treatment with sinularin, a natural product isolated from cultured soft coral possessing antineoplastic activity, at 12.5, 25, 50 microM resulted in cell cycle disruption and apoptosis in a dose-dependent manner in hepatocellular carcinoma cells [Chun et al., 2017]. The cell cycle disruption and apoptosis are induced by 30 micromol/L curcumin, a major component extracted from turmeric plants that have an anti-cancer effect [Liu et al., 2018].

Time-scale

MAA (5 mM) decreases CDK4, CDK2 expression 48 hrs after the treatment, which indicates the G_1 arrest [Parajuli et al., 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 et al., 2014].

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Relationship: 1735: Apoptosis leads to Spermatocyte depletion

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High

The apoptosis of the cells leads to spermatocyte depletion. The relationship between apoptosis and spermatocyte depletion is likely

well conserved between species. The examples are only given for mammals:

- Spermatogenesis was inhibited by the knockdown of Sucla2, a β subunit of succinyl coenzyme A synthase, via apoptosis in the mouse spermatocyte (*Mus musculus*) [Huang et al., 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 et al., 2011].
- MAA induced apoptosis and depletion of spermatocytes in adult rats (*Rattus norvegicus*) [Brinkworth et al., 1995].
- The apoptosis and proliferation inhibition induced by MAA, an HDAC inhibitor, was measured in human prostate cancer cell lines (*Homo sapiens*) [Parajuli et al., 2014].
- The cell viability inhibition induced by SAHA or TSA, which are HDAC inhibitors, was observed in NHDFs (*Homo sapiens*) [Glaser et al., 2003].
- The proliferation of the $HDAC^{-/-}$ ES cells was inhibited compared to $HDAC^{+/+}$ ES cells (*Homo sapiens*) [Zupkovitz et al., 2010].
- It has been reported that the 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 et al., 2001].
- The sperm counts in the cauda epididymis of rats exposed to butylparaben were significantly decreased (*Rattus norvegicus*) [Oishi, 2001].
- MAA treatment-induced spermatocyte death in Sprague-Dawley rats (*Rattus norvegicus*) [Wade et al., 2008].

Key Event Relationship Description

Apoptosis results in spermatocyte depletion via cell death. Apoptosis and spermatocyte depletion is correlated, where spermatocyte depletion via apoptosis is a general mechanism [Brinkworth et al., 1995].

Evidence Supporting this KER

Biological Plausibility

Induced apoptosis during the development of germ cells results in the progressive depletion of spermatocytes [Brinkworth et al., 1995]. An HDAC inhibitor, MAA, induced apoptosis and spermatocyte depletion at stages IX-II [Brinkworth et al., 1995].

Empirical Evidence

In the mouse spermatocyte, spermatogenesis is inhibited by knockdown of Sucla2, a beta subunit of succinyl coenzyme A synthase, which is located in mitochondria and catalyzes the reversible synthesis of succinate and adenosine triphosphate in the tricarboxylic acid cycle [Huang et al., 2016]. The knockdown of Sucla2 induces apoptosis of mouse spermatocytes [Huang et al., 2016]. The prolonged cryptorchidism leads to germs cell apoptosis and testicular sperm count decrease [Barqawi et al., 2004]. CD147 was reported to regulate apoptosis in mouse testis and spermatocyte cell line (GC-2 cells) via NFκB pathway [Wang et al., 2017]. 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 et al., 2011].

Uncertainties and Inconsistencies

The process of apoptosis is necessary for the meiosis 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].

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Relationship: 1734: Spermatocyte depletion leads to Testicular atrophy

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

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

Key Event Relationship Description

Spermatocyte depletion leads to testicular atrophy with a decrease in size. The spermatocyte depletion is involved in testicular atrophy and testicular toxicity [Chapin et al., 1984]. There are different insults that can induce spermatocyte depletion and consequently testicular atrophy.

Evidence Supporting this KER

Biological Plausibility

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

Empirical Evidence

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

Uncertainties and Inconsistencies

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

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List of Non Adjacent Key Event Relationships

Relationship: 1715: Histone deacetylase inhibition leads to Cell cycle, disrupted

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

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 et al., 2014].

Apicidin induced G_1 cell cycle arrest in HeLa cells (*Homo sapiens*) [Han et al., 2000].

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

Loss of HDAC1 in mouse embryonic stem (ES) cells results in the acetylation of histones H3 and H4, up-regulation of cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, and inhibition of proliferation *(Mus musculus)* [Lagger et al., 2002].

Key Event Relationship Description

HDAC inhibition leads to cell cycle arrest including G_1/S phase arrest [Falkenberg and Johnstone, 2014]. The HDAC inhibition-induced cell cycle arrest is mediated by transcriptional changes of the CDK inhibitors such as p21 [Falkenberg and Johnstone, 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 and Johnstone, 2014]. In leukemia, an oncogenic fusion protein recruits a variety of proteins including HDACs to repress cell cycle inhibitors, which suggests that HDAC inhibition leads to cell cycle dysregulation [Falkenberg and Johnstone, 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 et al., 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 et al., 2000]. Apicidin induced hyperacetylation of histone H4, up-regulation of p21, and G_0/G_1 cell cycle arrest in HeLa cells [Han et al., 2000].
- Falkenberg and Johnstone (2014) nicely reviewed that HDAC inhibition leads to cell cycle arrest in which G_1/S phase arrest occurs via up-regulation of p21.
- 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 et al., 2002].
- G_1/S transition blockade was observed in methoxyacetic acid (MAA)-treated prostate cancer cells [Parajuli et al., 2014].
- The change in the amounts of cells in the G_1 phase and S phase of the cell cycle was detected in mouse HDAC1 knock-out fibroblast lines [Zupkovitz et al., 2010].
- MAA, an HDI, induced cell cycle arrest and up-regulation of p21 expression and inhibited prostate cancer cell growth [Parajuli et al., 2014].

Uncertainties and Inconsistencies

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 et al., 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 et al., 2014]. Almost 80% of the cells were arrested in the G_1 phase upon stimulation of MAA, whereas approximately 40 to 60 % of the cells were in the G_1 phase without MAA treatment [Parajuli et al., 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 et al., 2014].

References

Falkenberg, K.J. and Johnstone, R.W. (2014), "Histone deacetylases and their inhibitors in cancer, neurological disease and immune disorders", Nat Rev Drug Discov 13:673-691

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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

Relationship: 1716: Histone deacetylase inhibition leads to Apoptosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

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 et al., 2016].

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

SAHA or TSA, which are HDAC inhibitors, reduced cell viability in NHDFs (*Homo sapiens*) [Glaser et al., 2003].

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

Key Event Relationship Description

HDAC inhibition leads to cell death through the apoptotic pathways [Falkenberg and Johnstone, 2014]. The intrinsic apoptosis pathway requires BH3-only proteins, and BCL-2 protein overexpression inhibits apoptosis [Falkenberg and Johnstone, 2014]. Administration of methoxyacetic acid (MAA), an HDAC inhibitor, causes apoptosis with DNA ladder in male germ cells [Brinkworth et al., 1995]. MAA induces the apoptosis of spermatocytes at spermatogenic cycle stage IX-II [Brinkworth et al., 1995].

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 and Johnstone, 2014].

Empirical Evidence

- MAA-induced spermatocyte death is associated with histone acetylation increase [Wade et al., 2008].
- The HDAC inhibition induced apoptosis markers such as BAK overexpression and suppression of phosphorylated AKT [Henderson et al., 2016].
- The administration of MAA can cause apoptosis in the germ cells of adult male rats [Brinkworth et al., 1995].

Uncertainties and Inconsistencies

It is uncertain through which pathway the HDAC inhibition induces apoptosis.

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 et al., 2014].

Time-scale

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

References

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

Falkenberg, K.J. and Johnstone, R.W. (2014), "Histone deacetylases and their inhibitors in cancer, neurological disease and immune disorders", Nat Rev Drug Discov 13:673-691

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

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

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

Relationship: 2010: Histone deacetylase inhibition leads to Spermatocyte depletion

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Male	High

Histone deacetylase inhibition by histone deacetylase inhibitors caused spermatocyte death in rats. MAA treatment induced spermatocyte death in Sprague-Dawley rats (*Rattus norvegicus*) [Wade et al., 2008].

VPA exposure caused a decrease in sperm count in humans (*Homo sapiens*) [Yerby and McCoy, 1999; Kose-Ozlece et al., 2015].

Key Event Relationship Description

Histone deacetylase inhibition triggered by histone deacetylase inhibitors such as methoxyacetic acid (MAA) leads to spermatocyte death causing spermatocyte depletion [Wade et al., 2008]. Histone deacetylase inhibition leads to an increase in histone acetylation, leading to spermatocyte apoptosis.

Evidence Supporting this KER

MAA administration induces spermatocyte deaths, which has been revealed by section staining of the germ cell death [Wade et al., 2008].

Biological Plausibility

Histone deacetylase inhibition causes histone acetylation, which increases the gene expression of cell-cyle-related proteins, followed by spermatocyte apoptosis in testis.

Empirical Evidence

Administration of MAA in rats, a histone deacetylase inhibitor (HDI), demonstrated the emergence of TUNEL-positive spermatocytes, which indicates spermatocyte apoptosis [Wade et al., 2008]. Treatment of valproate (VPA) resulted in a decline in the sperm count [Yerby and McCoy, 1999; Kose-Ozlece et al., 2015].

Quantitative Understanding of the Linkage

The administration of MAA in rats induced spermatocyte depletion which was confirmed with TUNEL-staining of the germ cells [Wade et al., 2008].

Time-scale

TUNEL-positive germ cells were increased after 8, 12, and 24 hrs of MAA exposure (650 mg/kg i.p.) in the rats [Wade et al., 2008]. TUNEL-positive zygotene spermatocytes have emerged after 12 hrs of MAA exposure in the rats, which was confirmed by the section staining [Wade et al., 2008].

References

Kose-Ozlece, H. et al. (2015), "Alterations in semen parameters in men with epilepsy treated with valproate", Iran J Neurol 14:164-167

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

Yerby, M.S. and McCoy, G.B. (1999), "Male infertility: Possible association with valproate exposure", Epilepsia 40:520-521

Relationship: 1717: Histone deacetylase inhibition leads to Testicular atrophy

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Histone deacetylase inhibition leading to testicular atrophy	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High

MAA induced spermatocyte apoptosis and cell morphology change in human testes (*Homo sapiens*) [Li et al., 1996].

Valproic acid caused the decrease in rat testicular weight (*Rattus norvegicus*) [Kallen, 2004].

Key Event Relationship Description

HDAC inhibition induced testicular toxicity including testis atrophy such as the decrease in size [Miller et al., 1982]. HDAC inhibition in cell culture resulted in testicular toxicity including germ cell apoptosis and cell morphology change [Li et al., 1996]. Valproic acid, an 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 et al., 1996]. The HDAC inhibitor treatment resulted in degeneration in spermatocytes in rat seminiferous tubules [Li et al., 1996]. The HDAC inhibition induced germ cell apoptosis in human testicular tissues [Li et al., 1996].

Empirical Evidence

- HDAC inhibitor, methoxyacetic acid (MAA), (300 mg/kg) induced testicular toxicity measured with testis weight loss [Miller et al., 1982].
- MAA induced apoptosis and degeneration in spermatocytes in human testicular tissue and 25-day rat seminiferous tubule cultures [Li et al., 1996].
- MAA-induced spermatocyte death with an association of histone acetylation increase [Wade et al., 2008].
- MAA-induced apoptosis in male germ cells was modulated by Sertoli cells via P/Q type voltage-operated calcium channels [Barone et al., 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 et al., 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 et al., 1985].

Uncertainties and Inconsistencies

It is reported that HDAC inhibition leads to teratogenic toxicity, whereas the correlation between testicular toxicity and teratogenic toxicity by HDAC inhibition is not fully understood [Menegola et al., 2006]. The oral administration of vorinostat (SAHA), an 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 et al., 2008]. Some studies have demonstrated that the decrease in histone acetylation in spermatids is associated with impaired spermatogenesis corresponding with the well-known reduction of protamine expression in the cells [Sonnack et al., 2002; Li et al., 2014]. It has also been reported that the histological examination of sections revealed no difference between wild-type and HDAC6-deficient testes [Zhang et al., 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 et al., 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 et al., 1984]. The treatment of 5 mM MAA for 5 hrs induced the pachytene spermatocyte death in early-stage tubules in 19 hrs [Li et al., 1996]. The degeneration in late spermatocytes was observed in late-stage tubules in 19 hrs after 5 mM MAA treatment for 5 hrs [Li et al., 1996].

References

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

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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

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

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

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