AOP ID and Title:

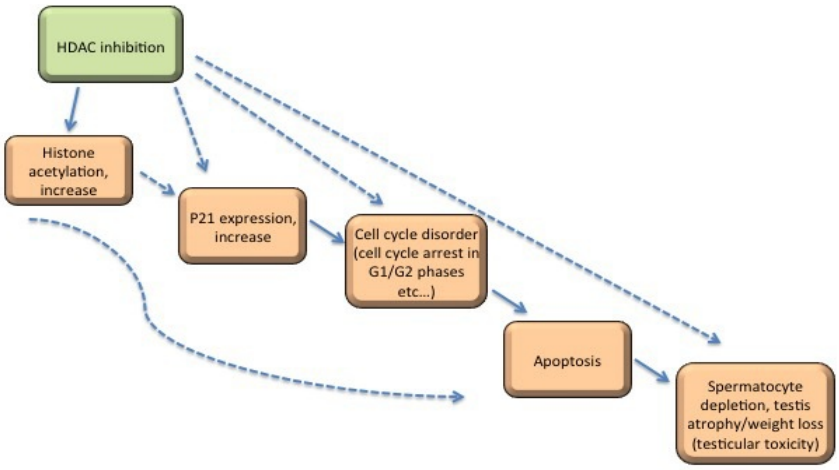
SNAPSHOT

Created at: 2018-02-23 15:17

AOP 212: Histone deacetylase inhibition leading to testicular toxicity

Short Title: Histone deacetylase inhibition leading to testicular toxicity

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.52	Included in OECD Work Plan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. Histone deacetylase (HDAC) inhibitors (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, methoxyacetic acid), 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), 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 molecular initiating event (MIE), histone acetylation increase, p21 expression increase, and cell cycle disorder as key events (KEs). Adverse outcome includes spermatocyte depletion and testis atrophy and weight loss. We propose new adverse outcome pathway (AOP) for histone deacetylase inhibition leading to testicular toxicity.

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 expression, increase (https://aopwiki.org/events/1504)	p21 expression, increase
4	KE	1505	cell cycle disorder (https://aopwiki.org/events/1505)	cell cycle disorder
5	KE	1262	Apoptosis (https://aopwiki.org/events/1262)	Apoptosis
6	AO	1506	spermatocyte depletion, testis atrophy/weight loss (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	High
Histone acetylation, increase (https://aopwiki.org/relationships/1710)	adjacent	p21 expression, increase	Moderate	Moderate
p21 expression, increase (https://aopwiki.org/relationships/1711)	adjacent	cell cycle disorder	High	Moderate
cell cycle disorder (https://aopwiki.org/relationships/1712)	adjacent	Apoptosis	High	Moderate
Apoptosis (https://aopwiki.org/relationships/1713)	adjacent	spermatocyte depletion, testis atrophy/weight loss (testicular toxicity)	High	Moderate
Histone deacetylase inhibition (https://aopwiki.org/relationships/1714)	non-adjacent	p21 expression, increase	High	High
Histone deacetylase inhibition (https://aopwiki.org/relationships/1715)	non-adjacent	cell cycle disorder	High	High
Histone deacetylase inhibition (https://aopwiki.org/relationships/1716)	non-adjacent	Apoptosis	High	High
Histone deacetylase inhibition (https://aopwiki.org/relationships/1717)	non-adjacent	spermatocyte depletion, testis atrophy/weight loss (testicular toxicity)	High	High

Stressors

Name	Evidence
Methoxyacetic acid	High
Butyrate	High
Trichostatin A	High

Overall Assessment of the AOP

Assessment of the Weight-of-Evidence supporting the AOP

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.

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). Other study showed that MAA induced up-regulation of p21 expression is mediated through histone hyperacetylation and independent of p53/p63/p73 (Parajuli).

NF-kappaB pathway

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).

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 SR and Liu Y).

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). Inhibition of 5,10-CH_2-THF production by MAA may decreases deoxynucleotide pool in spermatocytes (Yamazoe).

Assessment of the quantitative understanding of the AOP

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

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

Life stage applicability

Life stage	Evidence
Adult, reproductively mature	Strong

Taxonomic applicability

Name	Scientific name	Evidence
human	Homo sapiens	Moderate
mouse	Mus musculus	Moderate
rat	Rattus norvegicus	Strong

Sex applicability

Sex	Evidence
Male	Strong

Essentiality of the Key Events

Molecular Initiating Event	Support for essentiality
Histone deacetylase inhibition	Strong

Key Event	Support for essentiality
Histone acetylation, increase	Strong
p21 expression, increase	Moderate
Cell cycle disorder	Moderate

Apoptosis	Moderate

Weight of Evidence Summary

Event	Description	Triggers	Weight of Evidence
Histone deacetylase inhibition	Leads to	Histone acetylation, increase	Strong
Histone acetylation, increase	Leads to	p21 expression, increase	Strong
p21 expression, increase	Leads to	Cell cycle disorder	Strong
Cell cycle disorder	Leads to	Apoptosis	Strong
Apoptosis	Leads to	Spermatocyte depletion, Testis atrophy/weight loss (Testicular toxicity)	Moderate
Histone deacetylase inhibition	Indirectly Leads to	p21 expression, increase	Strong
Histone deacetylase inhibition	Indirectly Leads to	Cell cycle disorder	Strong
Histone deacetylase inhibition	Indirectly Leads to	Cell death, apoptosis	Strong
Histone deacetylase inhibition	Indirectly Leads to	Spermatocyte depletion, Testis atrophy/weight loss (Testicular toxicity)	Strong

Quantitative Consideration

Event	Description	Triggers	Quantitative understanding
Histone deacetylase inhibition	Leads to	Histone acetylation, increase	Strong
Histone acetylation, increase	Leads to	p21 expression, increase	Moderate
p21 expression, increase	Leads to	Cell cycle disorder	Moderate
Cell cycle disorder	Leads to	Apoptosis	Moderate
Apoptosis	Leads to	Spermatocyte depletion, Testis atrophy/weight loss (Testicular toxicity)	Moderate
Histone deacetylase inhibition	Indirectly Leads to	p21 expression, increase	Strong
Histone deacetylase inhibition	Indirectly Leads to	Cell cycle disorder	Strong

Histone deacetylase inhibition	Indirectly Leads to	Cell death, apoptosis	Strong
Histone deacetylase inhibition	Indirectly Leads to	Spermatocyte depletion, Testis atrophy/weight loss (Testicular toxicity)	Strong

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.

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Appendix 1

List of MIEs in this AOP

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

Short Name: Histone deacetylase inhibition

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

Stressors

Name
Methoxyacetic acid
Butyrate
Trichostatin A

Biological Context

Level of Biological Organization
Molecular

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 VM et al 2004, Ropero S and Esteller M, Villar-Garea et al]. There is a report showing that TSA and butyrate competitively inhibits HDAC activity [Sekhavat A]. HDIs inhibit preferentially HDACs with some selectiveness [Hu E et al]. 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 E et al]. TSA inhibits HDAC1, 2, 3 of class I HDACs. HDAC 1, 4, 6 are related to tumor size [Damaskos]. MAA (2 or 5 mM) inhibited HDAC activity in dose-response manner in rat testis cytosolic and nuclear extracts [Wade MG 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)

Name	Scientific Name	Tissue/Organ or Cell type	Evidence	Reference
Rat	Rattus norvegicus	Brown Norway rat (output: rate of resorption of subretinal blebs) (Desjardins) / Sprague-Dawley rat (Wade) / pc-12 cells (Steffan JS)	Strong	Desjardins D et al. Wade MG et al 2008 Steffan JS et al.
Human	Homo sapiens	Human (Ansari) / NSCLC cells (output: cell growth) (Miyanaga) / HeLa cell (Jansen MS, Di Renzo) / ARPE19 cells (Desjardins)	Strong	Ansari J et al., Miyanaga A et al. Jansen MS et al. 2004 Di Renzo et al. Desjardins D et al.
Mouse	Mus musculus	Splenocytes (Mishra) / brain, spleen (Hockly) / spleen (Jansen MS) / embyo (Di Renzo)	Strong	Mishra N et al. Hockly E et al. Jansen MS et al. 2004 Di Renzo et al.

Key Event Description

Site of action: The site of action for the molecular initiating event is the spermatocytes in testis.

The nucleosome consists of core histones having classes of H2A, H2B, H3 and H4) [Damaskos]. 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]. Histone deacetylase inhibitor (HDI) inhibits HDAC and acetylate the histones and release the DNA strand to induce the binding of transcription factors [Taunton et al]. 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 et al] [Minucci S].

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, Barneda-Zahonero]. HDACs 1, 2 and 3 are ubiquitously expressed, whereas HDAC8 is predominantly expressed in cells with smooth muscle/myoepithelial differentiation [Weichert]. HDAC6 is not observed to express in lymphocytes, stromal cells and vascular endothelial cells [Weichert]. Class III HDACs sirtuins are widely expressed and localized in different cellular compartments [Barneda-Zahonero]. SirT1 is highly expressed in testis, thymus and multiple types of germ cells [Bell]. HDAC11 expression is enriched in kidney, brain, testis, heart and skeletal muscle [Barneda-Zahonero].

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 VM 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 VM et al 2004]. Epigenetic modifications including the histone acetylation are measured using chromatin immunoprecipitation-microarray hybridization (ChIP-chip) [ENCODE Project Consortium, Ren B et al]. ChIP detects physical interaction between transcription factors or cofactors and the chromosome [Johnson DS et al].

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List of Key Events in the AOP

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

Short Name: Histone acetylation, increase

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

Domain of Applicability

MAA induces acetylation of histones H3 and H4 in Sprague-Dawley (Rattus norvegicus) [Wade MG]. It is also reporeted that MAA promotes acetylation of H4 in HeLa cells (Homo sapiens) and spleens from C57BL/6 mice (Mus musculus) treated with MAA [Jansen]. VPA induces hyperacetylation of histone H4 in protein extract of mouse embryos (Mus musculus) exposed in utero for 1h to VPA [Di Renzo]. Apicidin, MS-275 and sodium butyrate induce hyperacetylation of histone H4 in homogenates from mouse embryos (Mus musculus) after the treatments [Di Renzo]. MAA acetylates histones H3K9 and H4K12 in limbs of CD1 mice (Mus musculus) [Dayan C].

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 MG 2008].

How it is Measured or Detected

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

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

Short Name: p21 expression, increase

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

Domain of Applicability

FK228 up-regulated p21 level in human esophageal cancer TE2 cells (*Homo sapiens*) [Hoshino I]. MAA induced p21 up-regulation in human prostate cancer cell lines (*Homo sapiens*) [Parajuli]. MAA increases p21 expression in human bladder carcinoma cells, T24 (*Homo sapiens*) [Glaser]. MAA up-regulated p21 expression in limbs of CD1 embryonic mice (*Mus musculus*) [Dayan C].

Key Event Description

p21 (CDKN1A) 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. Acetylation of p21 promoter and p21 mRNA were correlated in valproic acid and analog treatments (Gurvich).

How it is Measured or Detected

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

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Event: 1505: cell cycle disorder (https://aopwiki.org/events/1505)

Short Name: cell cycle disorder

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

Domain of Applicability

MAA blocks G_1/S transition in human prostate cancer cell cycles (*Homo sapiens*) [Parajuli]. The change in the amounts of cells in G_1 phase and S phase of cell cycle was detected in mouse HDAC1$^{-/-}$ fibroblast lines (*Mus musculus*) [Zupkovitz].

Key Event Description

In spermatocytes, the mitosis and meiosis are activated and regulated. The dysregulation of cell cycle effects the spermatogenesis such as numbers. The phosphorylation of p21 regulates its function [Moussa, Child]. The up-regulation of p21 level in iron-chelated cancer cells was observed [Moussa]. G1/S transition blockade was observed in MAA-treated prostate cancer cells [Parajuli]. Valproic acid (VPA), a well-known teratogen, inhibits HDACs, which leads to the teratogenic effect [Gurvich].

How it is Measured or Detected

Cell cycle analysis was performed with cells treated with HDAC inhibitor [Parajuli]. 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 [Parajuli, Li Q]. Cell cycle distribution in HDAC1$^{-/-}$ fibroblast lines was also analyzed by fluorescence-activated cell sorter (FACS) analysis with a Partec PAS-II sorter [Zupkovitz]. The four cell cycle phases in living cells can be measured with four-color fluorescent proteins using live cell imaging [Bajar].

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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

Biological Context

Level of Biological Organization
Tissue

Domain of Applicability

The apoptosis and proliferation inhibition induced by MAA was measured in human prostate cancer cell lines (*Homo sapiens*) [Parajuli]. The cell viability inhibition induced by SAHA or TSA was observed in NHDFs (*Homo sapiens*) [Glaser]. The proliferation of the $HDAC^{-/-}$ ES cells was inhibited compared to $HDAC^{+/+}$ ES cells (*Homo sapiens*) [Zupkovitz].

Key Event Description

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]. MAA (5 mM or 20 mM) decreased BIRC2 protein expression and activated caspases 7 and 3 [Parajuli]. MAA induces apoptotic nucleosome increase and cleaved PARP protein expression [Parajuli]. MAA decreased cell viability of human prostate cancer cell lines [Parajuli]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation/viability of the cells [Glaser]. The impaired proliferation was observed in $HDAC^{-/-}$ ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz].

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

Apoptotic nucleosomes were detected using Cell Death Detection ELISA kit, which were calculated as absorbance subtraction at 405 nm and 490 [Parajuli]. Cell viability was measured with live cell number changes using the CellTiter-Glo Luminescent Cell Viability Assay [Parajuli]. Cleavage of PARP was detected with Western blotting [Parajuli]. The proliferation/viability of NHDFs was measured with Alamar-Blue [modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [Glaser]. Proliferation of the HDAC$^{-/-}$ ES cells was determined with crystal violet and measurement of absorbance at 595 nm [Zupkovitz].

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List of Adverse Outcomes in this AOP

Event: 1506: spermatocyte depletion, testis atrophy/weight loss (testicular toxicity) (https://aopwiki.org/events/1506)

Short Name: testicular toxicity

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

Domain of Applicability

It has been reported that mice lacking both *Ink4c* and *Ink4d* produced few mature sperm, and the residual spermatozoa had reduced motility and decreased viability (*Mus musculus*) [Zindy]. The sperm counts in the cauda epididymis of rats exposed to butylparaben were significantly decreased (*Rattus norvegicus*) [Oishi S2001]. EGME or MAA treatment induced the testicular damage in rat (*Rattus norvegicus*) [Foster PMD1983]. 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 D]. 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 WW]. EGME has effects in disruption of spermatogenesis in rabbits (*Oryctolagus cuniculus*) [Foote]. 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*) MAA treatment induces spermatocyte death in Sprague-Dawley rats (*Rattus norvegicus*) [Wade 2008]. Treatment of TSA resulted in a dose-dependent decrease in relative testis weight due to impaired spermatogenesis in mice, and impaired meiosis [Fenic 2004, 2008].

Key Event Description

Spermatocytes are differentiated from spermatogonial stem cells via random proliferation, differentiation and synchronized mitoses with several stages [Rooij]. Spermatogenesis is controlled by the cyclin D-dependent kinase inhibitors p18^{Ink4c} and p19^{Ink4d} [Zindy]. It is hypothesized that the testicular effects of 1,6-dimethoxyhexane (DMH) are caused by its metabolism to MAA [Wade2006, Poon2004]. MAA produces testicular and thymic atrophy [Miller1982, Moss1985]. 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].

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]. 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 S2001]. The weights of testes of MAA-treated rats were measured to detect the testicular atrophy [Foster

PMD1983]. 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 PMD1983]. In detail, the animals were *ip* injected with ^{65}Zn-Cl_2 in saline 2 days before treatment with either EGM or MAA. After the measurement of baseline values of ^{65}Zn urinary excretion, groups of animals were administered with EGME or an equimolar dose of MAA by oral gavage for 4 days and urine samples were collected daily [Foster PMD1983]. Twenty-four hours after the last dose of EGME or MAA, the animals were killed and the ^{65}Zn content in urine and tissues was measured with a Packard 5230 autogamma spectrometer [Foster PMD1983]. Testis were fixed in Bouin's fluid for light microscopy or diced and fixed in a 4% formaldehyde plus 1% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.29 for transmission electron microscopy [Foster PMD1983, McDowell and Trump1976]. Changes in sperm were measured by computer-assisted sperm analysis [Foote RH]. For the detection of apoptosis, the testes were fixed in modified Davidson fix for 48 hrs, postfixed for 24 hrs in 10% neutral buffered formalin, and embedded in paraffin. Germ cell death was visualized in testis sections by TUNEL staining [Wade2008]. The incidence of TUNEL-positive cells was expressed as the number of positive cells per tubule examined for one entire testis section per animal [Wade2008]. For the testis cell analysis, fresh testes were dispersed using a two-stage enzymatic digestion and incubated in BSA containing collagenase and DNase I [Wade2006]. The seminiferous tubules were further digested and cells were fixed in ice-cold 70% ethanol [Wade2006]. Relative proportions of spermatogenic cell populations were assessed in fixed cells using a flow cytometeric method [Wade2006]. 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. With the use of fluorescent probes to quantitatively label DNA (PI) and mitochondria (NAO), the relative numbers of cells in each intermediate population can be resolved to determine relatives at each germ cell stage. Cells were analyzed using a FACS Calibur flow cytometer fitted with an argon ion laser (488 nm line excitation); fluorescence emission of NAO was reflected by a 550 dichroic longpass filter and quantified after passage through a 530/30 nm bandpass filter [Wade2006]. Fluorescence of PI was detected after sequential passage through the 550 dichroic filter and a 670-nm longpass filter [Wade2006]. Relative proportions of cells in each population were calculated with WinList software [Wade2006]. The germ cell types in each population were identified under a fluorescent microscope (Zeiss Axioskop 2, Carl Zeiss, Thornwood, NY) by examination of cells from each population, which were isolated using the cell-sorting capacity of the FACS Calibur flow cytometer [Wade2006]. 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 [Wade2006]. 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 in 20 ml of STA solution (0.9% NaCl, 0.01% Triton X-100, and 0.025% sodium azide) [Wade2006]. 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 [Wade2006]. Homogenates of either tissue were then disrupted with a VibraCell sonicator using a microprobe [Wade2006]. Sperm or homogenization-resistant spermatid nuclei densities were calculated from the average number of nuclei in four fields on a Neubaur hemocytometer and were expressed as total or as per gram of epididymis or testis weight [Wade2006]. 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 at 340 nm in a reaction mixture that contained 4.2 mM of NAD in 10.5 mM Tris KCl (pH 9.0) at 30C [Wade2006].

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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 (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**	**High**

Evidence Supporting Applicability of this Relationship

Hyperacetylation by HDIs such as SAHA and Cpd-60 are observed in mouse (Schroeder et al). TSA induces acetylation of histone H4 in time-dependent manner in mouse cell lines (Alberts et al). AR-42, a novel HDI, induces hyperacetylation in human pancreatic cancer cells (Henderson et al.). SAHA and MS-275 hyperacetylates lysine of histones in human cell lines of epithelial (A549) and lymphoid origin (Jurkat) (Choudhary et al.). HDAC inhibitors, phenylbutyrate (PB) (2 mM) and TSA (200 nM) acetylate histones H3 and H4 in synovial cells from rats with adjuvant arthritis (Chung et al 2003). SAHA treatment induces the H3 and H4 histone acetylation in human corneal fibroblasts and conjunctiva from rabbits after glaucoma filtration surgery (Sharma A 2016). TSA induces the acetylation of histones H3 and H4 in *Brassica napus* microspore cultures (Li H et al 2014).

The inhibition of HDAC by HDIs is well conserved between species from lower organism to mammals [Richon VM 2004, Steffan JS et al, Desjardins D et al, Ansari J et al, Miyanaga A et al, Mishra N et al, Hockly E et al.] HDIs reduced lethality in *Drosophila* model and the HDAC activity was inhibited with HDIs in rat PC12 cells [Steffan JS]. 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 D et al]. HDIs were approved as drugs for multiple myeloma and T-cell lymphoma by FDA [Ansari]. HDIs inhibited cell growth in human non-small cell lung cancer cell lines [Miyanaga]. HDAC acetylation level was increased by HDIs in MRL-lpr/lpr murine model of lupus splenocytes [Mishra]. SAHA increased histone acetylation in brain and spleen of mice [Hockly]. MAA inhibits HDAC activity in HeLa cells and spleens from C57BL/6 mice [Jansen MS 2004]. It is also reported that MAA inhibits HDAC activity in testis cytosolic and nuclear extract of juvenile rats (27 days old) [Wade MG 2008]. VPA and TSA inhibit HDAC enzymatic activity in mouse embryo and human HeLa cell nuclear extract [Di Renzo].

Key Event Relationship Description

The gene transcription is regulated with the balance of acetylation. The histone acetylation leads to the gene transcription increase, whereas the deacetylation leads to the gene silencing in general. 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). HDAC inhibition by HDIs leads to hyperacetylation of histone and a large number of cellular proteins (Falkenberg 2014). Histone hyperacetylation by HDIs leads to transcriptional activation of genes (Falkenberg 2014). Measured properties of 20 valproic acid derivatives show that concentration of half-maximum inhibitory effect (IC_{50}) in the HDAC enzyme inhibition correlates with teratogenic potential, and HDAC inhibitory derivatives show hyperacetylation of core histone 4 in treated F9 cells (Eikel 2006).

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. It is likely that the post-translational modifications such as acetylation, phosphorylation and methylation regulates biological function, which suggests that the hyperacetylation induced by HDAC inhibition by HDIs leads to the regulation of gene transcription.

Empirical Evidence

HDIs increase histone acetylation in brain (Schroeder FA). 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). HDAC1 and HDAC3 activity are inhibited by *m*-carboxycinnamic acid bishydroxamide (CBHA) and suberoylanilide hydroxamic acid (SAHA) [Richon VM et al]. SAHA, inhibiting HDACs, leads to cause growth arrest, differentiation, and/or apoptosis of tumor, and it up-regulates thioredoxin-binding protein-2, whereas it down-regulates thioredoxin [Butler LM et al]. HDAC regulates gene expression of the regulators by deacetylating lysine residues of the transcription factors [Freiman RN and Tjian R]. HDAC activity is related to gene silencing, whereas histone acetyl transferase (HAT) induces histone acetylation leading to gene transcription [Ropero S and Esteller M]. MAA treatment in rats induced histone hyperacetylation in histones H3 and H4 [Wade MG]. MAA-induced spermatocyte death is associated with histone acetylation increase [Wade 2008]. The histone deacetylase (HDAC) inhibition induced by valproic acid (VPA) leads to histone hyperacetylation, followed by teratogenic toxicity [Eikel 2006]. Hyperacetylation of histone H3 in HDAC1-deficient ES cells was associated with proximal p21 promoter, while the distal promoter region was not shown to be associated [Lagger].

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).

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Relationship: 1710: Histone acetylation, increase leads to p21 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

TSA and sodium butyrate induced p21 mRNA expression in HT-29 human colon carcinoma cells (*Homo sapiens*) (Wu JT). Scriptaid, a HDI, up-regulated p21 mRNA expression in mouse embryonic kidney cells (*Mus musculus*) (Chen S).

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 N). Transient histone hyperacetylation was sufficient for the activation of p21 (Wu JT). TSA (0.3 mM) induced p21 mRNA expression in 1 hr, whereas the induction is repressed in 24 hrs after stimulation (Wu JT). On the other hand, butyrate and repetitive doses of TSA induced p21 mRNA expression in 24 hrs (Wu JT). Histone hyperacetylating agents butyrate and TSA induced p21 mRNA expression (Archer SY). HDAC1 overexpression blocked the induction of p21 by butyrate and TSA, indicating that the p21 induction is mediated by histone hyperacetylation (Archer SY). 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 VM2000).

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 (Richon2000 et al, Struhl K). 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 (Richon2000).

Empirical Evidence

HDIs induce p53-independent expression of p21 via Sp1 binding sites in the p21 promoter (Gartel). MAA up-regulates *p21* (cyclin dependent kinase inhibitor 1A; CDKN1A) gene expression in limbs of CD1 mice [Dayan C]. Considering that acetylation of p53 at K379 requires for p53 binding at the *p21* promoter region, p53K379 acetylation and induction of *p21* expression seemed correlated [Dayan C]. However, MAA did not increased p53, p63 and p73 level in PC-3, although MAA (5 or 20 mM) induces p21 mRNA level in prostate cancer cells including PC-3, which indicates that MAA-mediated p21 enhancement is independent of p53/p63/p73 [Parajuli]. On the other hand, 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, which suggests that MAA-mediated p21 transcription increases are correlated with HDAC inhibition [Parajuli]. HDIs such as SAHA, TSA and MS-275 up-regulates p21 gene expression in T24 bladder carcinoma cells, which also suggests that HDAC inhibition leads to p21 up-regulation [Glaser]. Cyclin-dependent kinase (CDK) inhibitors p21 and p27 are up-regulated in HDAC1-deficient embryonic stem (ES) cells [Lagger]. In $HDAC1^{-/-}$ mouse embryonic fibroblasts, p21 level increase is observed, which indicates that HDAC suppression leads to p21 up-regulation [Zupkovitz]. Moreover, the increased acetylation of histones H3 and H4 at both proximal and distant promoter regions of p21 was observed in $HDAC1^{-/-}$ mouse embryonic fibroblasts, suggesting that p21 increase is mediated by the loss of HDAC1 [Zupkovitz]. MAA inhibits HDAC1, HDAC2, and HDAC3 to increase the levels of acetylated histone H4 [Parajuli KR, Jansen MS]. Loss of HDAC1 in ES cells decreased proliferation and cyclin A- and cyclin E-associated kinase activity in the HDAC1 mutant cells [Lagger]. HDIs accumulated acetylation of histones and induced p21 protein and mRNA expression (Richon2000 et al, Wu et al). Hyperacetylation of histone H3 in HDAC1-deficient ES cells was associated with proximal p21 promoter, while the distal promoter region was not shown to be associated [Lagger].

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 (Han2001). Pretreatment with selective PKC inhibitors calphostin A and rottlerin suppressed the promoter activity of p21WAF1/Cip1 activated by apicidin (Han2001). 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 (Han2001). The p21 promoter activation through Sp1 sites induced by apicidin is thought to be independent of histone hyperacetylation (Han2001). The apicidin is suggested to histone hyperacetylation leading to the antiproliferative activity (Han2000). These results indicate the inconclusive discussion in the linkage between histone acetylation and p21 activation.

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Relationship: 1711: p21 expression, increase leads to cell cycle disorder (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

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). P21 regulates the E2F transcriptional activity to control cell cycle in human U2OS osteosarcoma cells (*Homo sapiens*) (Delavaine L and La Thangue NB). Cell cycle is regulated by p21 through cyclins and CDKs in mice (*Mus musculus*) (Sherr CJ and Roberts JM).

Key Event Relationship Description

Cell cycle regulation through p21 activation is demonstrated by the interactions of p21 with cyclins (Dotto). P21 interacts directly with cyclins through a conserved region in close to its N-terminus (amino acids 17-24; Cy1) (Dotto). P21 has second weak cyclin binding domain near its C-terminus region (amino acids 153-159), which overlaps with its PCNA binding domain (Dotto). 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). Kinase activity of cyclin-Cdk kinase was inhibited by Cy1 site of p21 that is important for the interaction of p21 with cyclin-Cdk complexes (Chen). The peptide containing Cy1 site inhibited the kinase activity of cyclin E-Cdk2 and cyclin A-Cdk2 (Chen). 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, Ogryzko]. P21 inhibits cyclin-dependent kinases and regulates cell cycle to promote cell cycle arrest.

Evidence Supporting this KER

Biological Plausibility

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). Hyperoxia inhibits DNA replication through p21 and histone H3 expression (O'Reilly). 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).

Empirical Evidence

TSA induces p21 expression leading to cell cycle arrest (Gartel). 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). SAHA induced p53-independent p21 expression and

apoptosis in myelomonocytic leukemia cells (Gartel). The SAHA-related lethality was increased by anti-sense p21, which indicates a protective role of p21 against SAHA-induced apoptosis (Gartel).

Uncertainties and Inconsistencies

The up-regulation of p21 signaling and in testicular germ cells was observed in diabetes (Kilarkaje N). The dual roles of p21 in cell cycle arrest and antiapoptotic effect in the testicular germ cells of diabetic rats are suggested (Kilarkaje N). TSA promotes apoptosis via HDAC inhibition and p53 signaling pathway activation [Deng Z et al]. It is suggested that furazolidone induces reactive oxygen species leading to suppression of p-AKT and p21, and induction of apoptosis (Deng). The anti-apoptotic effect of p21 is mediated by caspase-3 inhibition, which demonstrates the possibility of cell-cycle independent effect on apoptosis (Deng). 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). This result suggests that the direct enhancement of p21 gene expression is related to the alteration of the cell cycle distribution (Kang). It has been demonstrated that p21 induces apoptosis in human cervical cancer cell lines (Tsao), whereas p21 is implicated in apoptosis inhibition by blocking activation of caspase-3 or interacting with ASK1 (Gartel AL, Zhang J). The study of postnatal telomere indicated that dysfunction of premature telomere induces cell-cycle arrest through p21 activation in mammalian cardiomyocytes (Aix). The proposed model showed that telomere inactivation showed reduced regenerative capacity by p21 activation and cell cycle arrest (Aix). 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 AR). 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, Ogryzko]. G_1/S transition blockade was observed in MAA-treated prostate cancer cells [Parajuli].

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Relationship: 1712: cell cycle disorder 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**	**High**	**Moderate**

Evidence Supporting Applicability of this Relationship

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

Key Event Relationship Description

SEE BIOLOGICAL PLAUSIBILITY

Evidence Supporting this KER

Biological Plausibility

Apoptosis, also called as “physiological cell death”, is involved in cell turnover, physiological involution and atrophy of various tissues and organs (Kerr JFR). The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances (Kerr JFR). 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 CE). Cell cycles characterized by the DNA content changes regulate cell death and cell proliferation (Lynch MP). Cell gain and loss are balanced with mitosis and apoptosis (Cree). Apoptosis is mediated by caspase activation (Porter AG). 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 AG). The activation of caspase-3 leads to apoptotic chromatin condensation and DNA fragmentation (Porter AG). 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 TW). Sinularin induced caspases 8, 9, and 3, and pro-apoptotic protein Bax, whereas it decrease the anti-apoptotic Bcl-2 protein expression level (Chung TW). The $p21^{CIP1/Waf1}$ induces cell cycle arrest, whereas the up-regulation of p21 signaling in testicular germ cell cytoplasm is suggested to promote antiapoptotic effect for DNA damage-induced cell death in diabetes (Kilarkaje N). 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 R). The microRNA-497 activated caspases 9 and 3, and decreased Bcl2 and CCND2 (Wu R). CCND2 is an important cell cycle gene that induces G_1 arrest (Li L), and deregulated CCND2 is implicated in cell proliferation inhibition (Wu R, Mermelstein A, Dong Q).

Empirical Evidence

Cell cycle arrest such as G_1 arrest and G_1/S arrest are observed in apoptosis (Li L, Dong Q). microRNA-1 and microRNA-206 represses CCND2, while microRNA-29 represses CCND2 and induces G_1 arrest and apoptosis in rhabdomyosarcoma (Li L). The treatment with MAA in prostate cancer cells induced growth arrest and apoptosis [Parajuli].

Uncertainties and Inconsistencies

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 R).

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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: 1713: Apoptosis leads to testicular toxicity (https://aopwiki.org/relationships/1713)

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

Spermatogenesis was inhibited by knockdown of Sucla2 via apoptosis in the mouse spermatocyte (*Mus musculus*) (Huang). 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 et al).

Key Event Relationship Description

In the mouse spermatocyte, spermatogenesis was inhibited by knockdown of Sucla2 via apoptosis (Huang). CD147 was reported to regulate apoptosis in mouse testis and spermatocyte cell line (GC-2 cells) via NFkB pathway (Wang).

Evidence Supporting this KER

Biological Plausibility
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 JFR et al).

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 Z et al).

Uncertainties and Inconsistencies
Spermatogonial stem cell self-renewal and spermatocyte meiosis are regulated by Sertoli cell signaling, which suggests us that various pathways other than HDAC inhibition in spermatocytes or spermatogonia are involved in the spermatocyte deletion and testis atrophy/weight loss (Chen SR). It should be noted that 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 M).

References

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

List of Non Adjacent Key Event Relationships

Relationship: 1714: Histone deacetylase inhibition leads to p21 expression, increase (https://aopwiki.org/relationships/1714)

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**	**High**

Evidence Supporting Applicability of this Relationship

The exposure of 2-MAA on mouse limbs in vitro induced histone hyperacetylation and p21 expression increase (*Mus musculus*) (Dayan). HDAC-deficient embryonic stem cells demonstrated up-regulation of p21 in mice (*Mus musculus*) (Lagger). HDAC inhibitor, AR-42 induced histone hyperacetylation and p21 up-regulation in human pancreatic cancer cells (*Homo sapiens*) (Henderson).

Key Event Relationship Description

HDAC inhibition leads to histone hyperacetylation and p21 activation (Falkenberg KJ, 2014). HDIs-induced G_1/S phase arrest occurs primarily through transcriptional changes in cell cycle regulatory genes, such as induction of the CDK inhibitors p21 (CDKN1A), $p15^{INK4B}$ (CDKN2B), $p19^{INK4D}$ (CDKN2D) and p57 (CDKN1C) (Falkenberg 2014). HDAC1-deficient embryonic stem cells showed decrease in cyclin-associated kinase activities and increase in $p21^{WAF1/CIP1}$ (Lagger G). Expression of p21 is up-regulated in HDAC1-null embryos (Lagger G). The HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), and MS-27-275 induced histone hyperacetylation and p21 up-regulation (Glaser). MAA up-regulated p21 mRNA level and acetylation of histone H3 and H4 (Parajuli KR).

Evidence Supporting this KER

Biological Plausibility

In human pancreatic cancer cell lines, p21 up-regulation and histone H3 hyperacetylation by HDAC inhibitor AR-42 were observed (Henderson). Furthermore, the oral administration of AR-42 at 50 mg/kg resulted in suppression of tumor in the pancreatic cancer cell xenograft and transgenic $KP^{fl/fl}C$ (LSL-$Kras^{G12D}$; $Trp53^{flox/flox}$; Pdx-1-Cre) mouse model (Henderson). The exposure of 2-methoxyacetic acid (2-MAA) induced morphological changes on embryonic forelimbs (Dayan).

Empirical Evidence

The expression of p21 was up-regulated with 10 mM of 2-MAA, whereas ethylene glycol monomethyl ether (EGME) did not increase p21 expression (Dayan). The treatment of 2-MAA induced histone acetylation in H3K9Ac and H4K12Ac, as well as p53K379Ac (Dayan).

Uncertainties and Inconsistencies

The exposure to 2-MAA induced p53 acetylation independent of histone hyperacetylation, which demonstrates the possibility in which several pathways are activated in HDAC inhibition towards apoptosis (Dayan). It has also been shown that p21 up-regulation by sodium butyrate is p53 dependent using the Chromatin Immunoprecipitation (ChIP) assay with anti-p53 monoclonal antibody (Saldanha). ST2783, a HDI, induced acetylation of p53 (Zuco). The mechanism inducing p21 by HDAC inhibition contains several pathway cross-talk.

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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

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

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

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Relationship: 1715: Histone deacetylase inhibition leads to cell cycle disorder (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**	**High**

Evidence Supporting Applicability of this Relationship

MAA induced G_1 cell cycle arrest in human prostate cancer cells (*Homo sapiens*) (Parajuli). Apicidin-induced G_1 cell cycle arrest in HeLa cells (*Homo sapiens*) (Han).

Key Event Relationship Description

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 JW). Apicidin induced hyperacetylation of histone H4, up-regulation of p21, and G_0/G_1 cell cycle arrest in HeLa cells (Han JW). HDAC inhibition leads to cell cycle arrest, where G_1/S phase arrest occurs with up-regulation of p21 (Falkenberg).

Evidence Supporting this KER

Biological Plausibility

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).

Empirical Evidence
In $HDAC1^{-/-}$ fibroblast lines, increase in the amount of cells in G_1 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]. 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).

Uncertainties and Inconsistencies
MAA, a HDI, induced cell cycle arrest and up-regulation of p21 expression, and inhibited prostate cancer cell growth (Parajuli). 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).

References

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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

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**	**High**	**High**

Evidence Supporting Applicability of this Relationship

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

Key Event Relationship Description

HDAC inhibition leads to cell death through the apoptotic pathways (Falkenberg). Intrinsic apoptosis pathway requires BH3-only proteins, and BCL-2 protein overexpression inhibits apoptosis (Falkenberg). 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). AR-42 inhibited the expression of $BCL\text{-}X_L$, an anti-apoptotic molecule in AsPC-1 tumor homogenates collected after 21 days of treatment (Henderson).

Evidence Supporting this KER

Biological Plausibility
Oral administration of AR-42, a HDAC inhibitor, at 50 mg/kg in human pancreatic cancer cell AsPC-1 xenograft and $KP^{fl/fl}C$ models resulted in the suppression of tumor (Henderson).

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). HDAC-null embryoid bodies showed a reduced inner cell mass and reduced colony formation (Lagger). HDAC inhibition by suberoylanilide hydroxamic acid (SAHA) inhibited proliferation of normal human dermal fibroblasts (NHDF) (Glaser).

Uncertainties and Inconsistencies
Methoxyacetic acid (MAA), a HDAC inhibitor, induced cell cycle arrest, apoptosis, leading to suppression of human prostate cancer cell growth (Parajuli). It is not fully elucidated whether MAA-induced apoptosis is involved in p53/p63/p73 pathway (Parajuli).

References

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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

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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

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**	**High**	**High**

Evidence Supporting Applicability of this Relationship

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).

Key Event Relationship Description

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, Yamazoe). Doxorubicin-resistant A549 cells showed reduced expression of HDAC1, 3 and 4 compared to A549 cells (El-Awady). Methoxyacetic acid (MAA)-induced apoptosis in male germ cells was modulated by Sertoli cells via P/Q type voltage-operated calcium channels (Barone F).

Evidence Supporting this KER

Biological Plausibility
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 PMD 1983). The investigation of 2-methoxyethanol (2-ME)-induced testicular toxicity has revealed that the conversion of 2-ME to methoxyacetic acid (MAA) is required in 2-ME-induced testicular toxicity (Moss).

Empirical Evidence
MAA (300 mg/kg) induced body weight loss and testicular toxicity measured with testis weight loss (Miller). MAA induced apoptosis and degeneration in spermatocytes in human testicular tissue and 25-day rat seminiferous tubule cultures (Li).

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). 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).

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