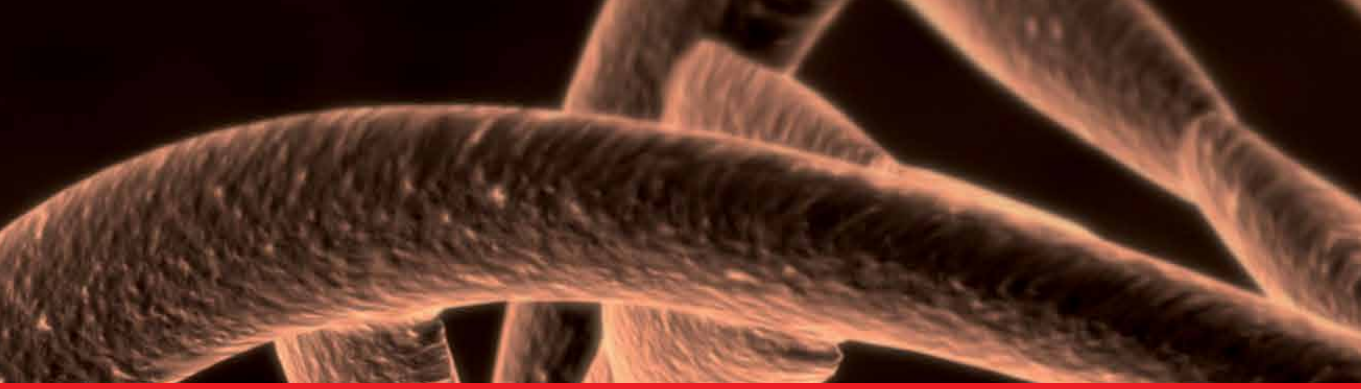

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Genotoxicity and Mutagenicity

Mechanisms and Test Methods

Edited by Sonia Soloneski and Marcelo L. Larramendy

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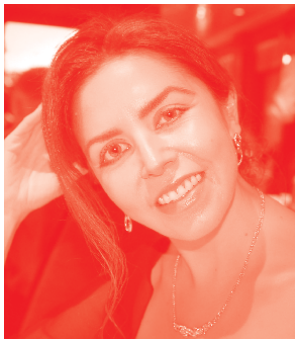

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Meet the editors

Sonia Soloneski has a Ph.D. in Natural Sciences and is an Assistant Professor of Molecular Cell Biology at the School of Natural Sciences and Museum of La Plata, National University of La Plata, Argentina. She is a member of the National Scientific and Technological Research Council (CONICET) of Argentina in the genetic toxicology field, the Latin American Association of Environmental Mutagenesis, Teratogenesis, and Carcinogenesis (ALAMCTA), the Argentinean Society of Toxicology (ATA), the Argentinean Society of Genetics (SAG), the Argentinean Society of Biology (SAB), and the Society of Environmental Toxicology and Chemistry (SETAC). She has authored more than 380 contributions in the field, including scientific publications in peer-reviewed journals and research communications. She has served as a review member for more than 30 scientific international journals. She has been a plenary speaker in scientific conferences and a member of scientific committees. She is a specialist in issues related to genetic toxicology, mutagenesis, and ecotoxicology.

Marcelo L. Larramendy, Ph.D., serves as a Professor of Molecular Cell Biology at the School of Natural Sciences and Museum (National University of La Plata, Argentina). He was appointed as Senior Researcher of the National Scientific and Technological Research Council of Argentina. He is a former member of the Executive Committee of the Latin American Association of Environmental Mutagenesis, Teratogenesis, and Carcinogenesis. He is the author of more than 450 contributions, including scientific publications, research communications, and conferences worldwide. He is the recipient of several national and international awards. Prof. Larramendy is a regular lecturer at the international A. Hollaender courses organized by the IAEMS and a former guest scientist at NIH (USA) and the University of Helsinki, (Finland). He is an expert in genetic toxicology and is, or has been, a referee for more than 20 international scientific journals. He was a member of the International Panel of Experts at the International Agency for Research on Cancer (IARC, WHO, Lyon, France) in 2015 for the evaluation of DDT, 2,4-D, and Lindane. Presently, Prof. Dr. Larramendy is Head of the Laboratory of Molecular Cytogenetics and Genotoxicology at the UNLP.

Contents

Preface

Organisms living in the real world are inevitably exposed to many chemical, physical, and biological agents that are harmful: food additives, natural toxins, pesticides, nanomaterials, metals, radiation, and viruses, among others. However, most of these agents, if not all, may have unexpected consequences on the biota. Organisms are continuously exposed to heterogeneous xenobiotics released into different habitats either deliberately, inadvertently, or through non-regulated industrial discharges. Understanding how these agents can produce genetic alterations in DNA and what their role is in different biological systems continue to receive intense attention in fields such as health, pharmaceutical, environment, industry, agriculture, and food sectors.

Mutagenicity denotes the generation of stable changes in the DNA molecule that differ from the normal sequence of an organism, which may result in a transmissible change in the genotype of living organisms. Any damaged genetic material could result in mutations, thus stimulating carcinogenic progression or establishing a framework for hereditary disorders. Whereas mutations are generated mainly by exogenous agents, named mutagens, the term genotoxic describes the capability of those chemical, physical, and biological agents to directly affect the structure of DNA, the cellular spindle apparatus, and/or the topoisomerase enzymes that modulate DNA topology during DNA replication as well as chromosome segregation, which are, finally, responsible for the fidelity of the genome. However, genotoxic damage to DNA is not always associated with mutations. Spontaneous mutations arise from a variety of sources due to errors in DNA replication, repair, and recombination, and the presence of transposable genetic elements. Many agents can produce chemically reactive species during their metabolism, or are themselves reactive and may, therefore, cause irreversible changes to DNA.

Heritable changes are the origin of innate metabolic deficiencies in cellular systems, generating morbidity and mortality in organisms. Genetic disorders can be produced by a mutation in only one or in multiple genes, through a combination of gene mutations and environmental factors or by damage at the chromosomal level that affects the number and/or structure of entire chromosome(s), or parts thereof. Mutations in cells are not only involved in the initiation and promotion of several human diseases, including cancer, but are also implicated in several genetic disorders, like anaemia, diabetes, cardiovascular alterations, obesity, atherosclerosis, and numerous other degenerative disorders. Currently, scientists recognize more than 4,000 human diseases that are produced by mutations as a result of a combinatorial failure of more than one of these processes.

As indicated in a book we published some years ago, entitled “Genotoxicity-A Predictable Risk To Our Actual World”, without knowledge of the mutagenic and genotoxic properties of chemical, physical, and biological agents, the evaluation of responses in living organisms, including humans, is difficult, and consequently the regulation of genotoxicants is a complex and difficult process. Accurate identification of the different classes of environmental genotoxicants and mutagens

would permit international regulatory scientific agencies to use this information in a variety of legislative decisions to establish priorities of public and scientific concern.

We have attempted to compile information from different fields, highlighting the detrimental influence that mutagenic and genotoxic agents inflict on DNA and how antimutagenic and anticarcinogenic modulators are able to reduce the negative impact of such toxic agents on living species. Antimutagens and anticarcinogens are agents that decrease the number of mutations in cells, modulating host defence mechanisms. Therefore, knowledge regarding the mechanism of action of potentially mutagenic and/or carcinogenic agents provides the basis for elucidation of how these protective chemicals exert a response. Antimutagens are employed as one of the key methods to increase cellular resistance to mutagens. They are able to reduce or even remove the mutagenic effects exerted by toxic xenobiotics, stimulating compensatory repair and tolerance pathways in the DNA. In regard to their mode of action, antimutagens can act by influencing different targets, such as cellular membranes, DNA damage repair, replication, chromatin organization, and cell signalling.

This book opens with an interesting discussion about the use of yeast as a model organism for studying the biological effects of the P450-mediated metabolism of xenobiotics. This chapter also focuses on strategies for employing multiple genetic endpoints in screening chemicals, yeast strains that facilitate phenotyping cytochrome P450 polymorphisms to test the safety of thousands of chemicals, the limitations of animal systems, the advantages of model organisms, and the humanization of yeast cells by expressing human cytochrome P450 genes. The second chapter describes a possible molecular mechanism for how the addition of exogenous polyamines may increase the production of improved strains of filamentous fungi and the biotechnological applications of this phenomenon. The third chapter provides information on chemical and physical mutagenesis in breeding, exemplified by new modern homozygous self-pollinated sunflower lines, as well as additional recommendations on the use of methods to induce mutagenesis, including methods of generation, investigation, and subsequent use of mutations. The fourth chapter comprises an excellent review comparing the specific toxicity and genotoxicity exerted by heavy metals such as lead and cadmium using mammalian cells as a biological matrix in the context of ecotoxicology. The fifth chapter describes the importance of doublecortin-like kinase 1 (DCLK1), a member of the protein kinase superfamily and the doublecortin family, and its role in DNA damage response and repair, via direct and indirect mechanisms. It is well known that DCLK1 is expressed in cancer stem cells, and is implicated in initiating tumours. The sixth chapter reviews the role of oxidative stress induced by vanadium (a common mechanism of action of metal pollutants), observed in in vivo and in vitro systems, highlighting the way the production of free radicals inflicts damage in biomolecules including DNA, proteins, lipids, and carbohydrates. In addition, the chapter emphasizes the protective role of two water-soluble antioxidants, namely carnosine and ascorbate, present in biological systems. The seventh chapter constitutes an update on how the w-/w+ somatic mutation and recombination test of Drosophila melanogaster are employed extensively for antigenotoxicity analysis, focusing on actual published results to aid in the development of a reliable protocol in antigenotoxicity. Finally, this book comprises a chapter discussing the properties of antimutagenic substances with multiple mechanisms of action, in addition to introducing different aspects of natural and synthetic antimutagens.

Further, the chapter includes a brief compilation of scientific findings, either from dietary sources or synthetic agents, with potential to combat the disorders caused by the mutagenic agents, noting possible future perspectives and mechanisms of antimutagenics.

The editors of Genotoxicity and Mutagenicity - Mechanisms and Test Methods are enormously grateful to all contributing authors for sharing their knowledge and insights in this book. They have made an extensive effort to gather the information included in every chapter. Readers are challenged to interpret the significance of various mechanisms and tested methodologies for detecting the causes and consequences of mutagenic and genotoxic agents. We hope that the topics discussed here encourage all those interested to explore new aspects of the fields of mutagenesis and genotoxicity by stimulating scientific dialogue. The publication of this book is of great importance to scientists, biologists, pharmacologists, physicians, and veterinarians, as well as engineers, teachers, graduate students, and administrators of environmental programmes, who can make use of these investigations to understand some aspects of mutagenic and genotoxic issues, making this volume a valuable reference in the future.

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Chapter 1

Genotoxic Assays for Measuring P450 Activation of Chemical Mutagens

Michael Fasullo

Abstract

This review discusses using yeast as a model organism for studying the biological effects of P450-mediated metabolism of xenobiotics. We discuss the challenges of testing the safety of thousands of chemicals currently introduced into the market place, the limitations of the animal systems, the advantages of model organisms, and the humanization of the yeast cells by expressing human cytochrome P450 (CYP) genes. We discuss strategies in utilizing multiple genetic endpoints in screening chemicals and yeast strains that facilitate phenotyping CYP polymorphisms. In particular, we discuss yeast mutants that facilitate xenobiotic import and retention and particular DNA repair mutants that can facilitate in measuring genotoxic endpoints and elucidating genotoxic mechanisms. New directions in toxicogenetics suggest that particular DNA damaging agents may interact with chromatin and perturb gene silencing, which may also generate genetic instabilities. By introducing human CYP genes into yeast strains, new strategies can be explored for high-throughput testing of xenobiotics and identifying potent DNA damaging agents.

Keywords: cytochrome P450 polymorphisms, genotoxins, budding yeast, recombination assays

1. Introduction

Genotoxins are generally referred to as chemical agents that cause DNA damage, which, in turn, can initiate recombination or mutation events or chromosome loss [1]. While mutagens and recombinagens are genotoxic, not all genotoxins are directly mutagenic [2]. Genotoxic exposure has been correlated to birth defects [3], cardiovascular disease [4], carcinogenesis [5], and accelerated aging [6]. Public health depends on minimizing exposure to genotoxic chemicals. Nonetheless, thousands of chemicals have yet to be tested, and new chemicals are annually synthesized. Federal agencies mandate that all chemicals be tested for safety before being introduced into the marketplace [7]. Generally, this testing has involved rapid screens for bacterial mutagenesis, micronuclei assays or comet assays for testing DNA fragmentation, and animal testing for determining carcinogenicity. Animal testing is often expensive and time-consuming and has increasingly raised ethical concerns. While microbial plate assays, such as the Ames test [8], have been standard in identifying chemical mutagens, some chemicals that test negative in

the Ames assays are carcinogenic, while others that test positive in the Ames assays are not carcinogens [8, 9]. Many chemicals are not genotoxic per se but require metabolic bioactivation [10]. The bioactivated compound is generally a highly reactive intermediate in a pathway which renders hydrophobic compounds more hydrophilic to facilitate excretion. While bioactivation does occur in specific animal models, toxicity outcomes differ depending on the species [11]. Thus, there is a need for metabolic competent cell-based assays that can measure multiple genotoxic endpoints.

Bioactivation occurs by phase I and phase II enzymes; phase I enzymes generally hydroxylate compounds so that phase II enzymes can conjugate larger molecules, facilitating the export and excretion of the modified compound. Phase I enzymes include cytochrome P450 monooxygenases (CYPs), which compose a superfamily of over 50 genes, and catalyze the formation of highly reactive electrophiles and epoxides, as intermediates in xenobiotic metabolism [12, 13]. Up to 80% of all bioactivations require CYPs [14]. For catalytic efficiency, the CYP proteins must be reduced by oxidoreductases, which are colocalized with CYPs in the endoplasmic reticulum (ER [15]).

Saccharomyces cerevisiae (budding yeast) is an excellent eukaryotic model organism for studying the genotoxicity of xenobiotics, including pharmaceuticals, pesticides, insecticides, and suspected carcinogens. Similar to bacterial organisms, yeast strains are easy to culture, grow rapidly, and can be manipulated genetically, rendering it possible to perform high-throughput analysis [16]. Many yeast genes are similar to human genes, and approximately 30% of can be functionally replaced by the human orthologue [17, 18]. DNA repair pathways and genes are also similar [17, 16]. Mitochondrial genotoxicity can also be measured [19]. Thus, identifying genotoxins and understanding their mechanisms in budding yeast can elucidate whether similar mechanisms occur in human cells.

However, yeasts also have some disadvantages. First, yeast cells contain a cell wall that blocks entry to carcinogens, and higher chemical concentrations are required in yeast than in mammalian organisms to observe similar genotoxic endpoints [1, 20]. Second, yeast lacks some functions of mammalian cells; while there are many yeast genes that have human homologs, other human DNA repair genes, such as p53, BRCA1, and BRCA2, have no corresponding yeast homologs. Nonetheless, the ability to modify the yeast genome has enabled yeast biologists to enhance carcinogen uptake and retention in cells [20, 21].

Engineered yeast strains enable high-throughput screens for identifying genotoxins among the thousands of novel synthetic chemicals, circumventing the limitations and reducing the escalating costs of animal tests. By expressing specific human cytochrome P450 genes in the engineered strains, tissue-specific metabolic activation can be simulated. Besides identifying genotoxins, engineered yeast strains can elucidate genotoxic mechanisms by measuring multiple genetic alterations, as well as DNA and organelle damage. Future engineering of yeast strains may identify additional human metabolic genes that can confer resistance to P450-activated genotoxins.

This review will address (1) characterization of human CYPs that activate the majority of carcinogens, (2) yeast vectors that have been engineered to express these CYPs, (3) plate and reporter assays that have been used to detect CYP-dependent activated compounds in yeast, (4) chemicals which have been identified and mechanistic insights that have been garnered by utilizing yeast genetics, (5) studies that have phenotypes P450 polymorphisms, (6) comparisons with other model eukaryotes, and (7) future directions in guiding genotoxic assays.

2. Phase I and phase II enzymes that bioactivate xenobiotics

While 57 CYPs have been identified, approximately 80% of all bioactivation is mediated by just 7 CYPs: CYP1A1, 1A2, 1B1, 2A13, 2A6, 2E1, and 3A4 [22]. Xenobiotic chemicals that are activated by these CYPs include polycyclic aromatic hydrocarbons (PAHs), aryl- and heterocyclic amines (HAAs), and nitrosamines, as well as small molecules such as benzene, naphthalene, and furans [22]. Examples of CYP-activated xenobiotics include tobacco carcinogens, industrial solvents, and food carcinogens, including the most potent liver carcinogen, aflatoxin B1 (AFB1). The importance of individual CYPs is underscored by observations that particular knockout mice are more resistant to environmental carcinogens, for example, fewer tumors arise in *Cyp1b1*$^{-/-}$ and *Cyp2a5* $^{-/-}$ knockout mice after exposure to 7,12-dimethylbenz[a]anthracene [23] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [24], respectively.

Phase II enzymes include glutathione S-transferases, N-acetyl transferases, epoxide hydrolases, glucuronidases, and sulfotransferases. They serve to both inactivate highly reactive intermediates that are formed by phase I enzymes and conjugate larger molecules onto the products of phase I reactions to facilitate export and excretion [25]. While some phase II enzymes, such as glutathione S-transferases (GSTs), may inactivate epoxide intermediates, other phase II enzymes, such as N-acetyl transferases (NATs), may facilitate the conversion of hydroxylated heterocyclic aromatic amines to highly nitrenium ions (**Figure 1** [25]). For example, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is hydroxylated by CYP1A2 in the liver [26]. Further modification by NAT2 acetylates the hydroxylated product, resulting in an unstable intermediate yielding a reactive nitrenium ion; this nitrenium ion reacts with DNA yielding deoxyguanine DNA adducts [25, 26]. Pathways by which activated HAAs drive colon carcinogenesis are unclear. One notion is that reactive IQ metabolites can also be glucuronidated in the liver and excreted into

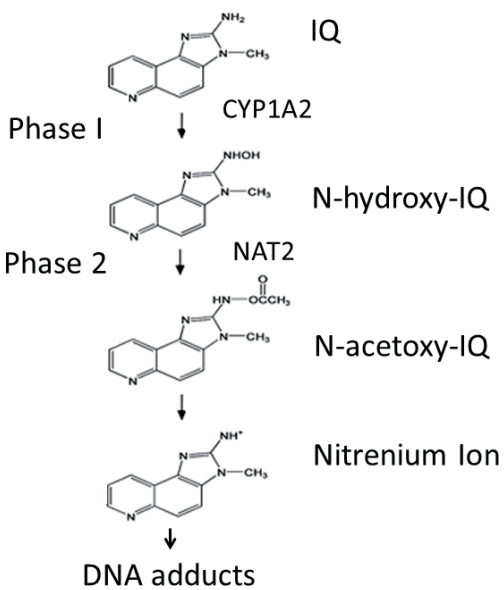

Figure 1.
Phase 1 and phase 2 metabolism of IQ. CYP1A2 generates the formation of N-hydroxy IQ. NAT2 generates an unstable molecule, N-acetoxy-IQ, which then generates a reactive nitrenium ion. The reactive nitrenium ion interacts with DNA to form adducts, particularly at the C8 and C2 positions of guanine. Figure was adapted from Kim and Guengerich [25].

the colon. Gut microbial glucuronidases then cleave the glucose and reactivate the compound leading to genotoxicity in the colon [27]. An alternative notion is that IQ is bioactivated to a mutagen in situ in the colon. Expression of multiple mammalian phase I and phase II enzymes may be important in rendering some compounds genotoxic; examples include CYP1A1 and epoxide hydrolase in the activation of benzo[a]pyrene and CYP2E1 and SULT1A1 for the activation of 2,5-dimethylfuran (DMF) and furfuryl alcohol (FFA) [5, 22].

When compounds are substrates for multiple CYPs or phase II enzymes, products of varying toxicity can be generated; examples of substrates include estradiol, N-acetyl-p-aminophenol (APAP or acetaminophen), and AFB1. In the case of estradiol, CYP1A2 and CYP3A4 predominately hydroxylate estradiol in the 2′ position generating 2′ hydroxyestradiol [28, 29], while CYP1B1 hydroxylates estradiol in the 4′ position generating 4′ hydroxyestradiol; further modification of 4′-hydroxyestradiol by peroxidases generates a highly reactive form that generates DNA adducts, while 2′ hydroxylestradiol can be detoxified [29]. In the case of acetaminophen, most acetaminophen is converted to nontoxic forms by sulfotransferases and glucuronidases; CYP2E1 converts acetaminophen to N-acetyl-*p*-benzoquinone imine (NAPQI), which depletes glutathione levels, causes oxidative stress, and is highly toxic in the liver [30]. Since CYP2E1 is induced by alcohol, the combination of alcohol and acetaminophen can be lethal [29]. Regarding AFB1, AFB1 is metabolized by CYP1A2 and CYP3A4 into a toxic epoxide intermediate, while the extrahepatic CYP1A1 can also convert AFB1 to AFM, which is still genotoxic but not as carcinogenic [31]. Because expression and inducibility of CYPs vary among individuals and multiple CYPs are expressed in tissues, it can be difficult to identify which CYP(s) generates the activated genotoxin.

Yeast presents advantages in deciphering which human CYPs can metabolize genotoxins. First, the three endogenous yeast CYPs largely function to synthesize ergosterol or dityrosine synthesis [32, 33]. Second, expression of CYPs in yeast can be regulated by inducible promoters or by copy number, mitigating potential toxic effects of their expression [34, 35]. Considering that CYP proteins locate to the yeast ER, the entire CYP cDNA can be expressed without truncating the sequence that encodes the N-terminus, as it is necessary for efficient CYP expression in *Escherichia coli* [36]. Third, although CYPs are differentially degraded [37], they are sufficiently stable to activate carcinogens for extended time duration, circumventing problems of transient or variable expression observed in cultures derived from cryopreserved hepatocytes. Based on genotoxic endpoints that can be easily scored, it is possible to phenotype CYP polymorphisms and to determine whether their substrate specificities are altered.

2.1 Mammalian CYP expression in budding yeast

Yeast has been an attractive organism for the expression of heterologous proteins and useful for characterizing biochemical properties of mammalian cytochrome P450 properties. Its success at producing large quantities of human proteins, such as human insulin [38], has largely been due to an advanced understanding of both the transcriptional and translational machinery of eukaryotic gene expression, including well-characterized transcriptional promoters and terminators [39]. Constitutive promoters for expression include *ADH1*, *GAPDH*, *PGK1*, *TPI*, *ENO*, *PYK1*, and *TEF*, while inducible promoters include *GAL1–10*, *CUP1*, *PHO5*, and *ADH* [40]. Expression can be further amplified by high-copy-number vectors or modulated by single-copy vectors, which have been well-described in the literature [34]. Oeda et al. [41] expressed rat CYP2E1cDNA using the constitutive *ADH1* promoter and the phosphoglycerol kinase (pGK) terminator using a high-copy-number vector.

The ability to bind carbon monoxide (CO) confirmed the presence of the recombinant protein. Characterization of human CYP3A4 produced in yeast underscored its broad substrate specificity [42, 43]. Additional in vitro studies involved expressing either human or rat cDNAs of CYP1A1, CYP2E1, CYP1A2, and CYP1B1 and confirming their biochemical properties [43]. A list of CYPs that have been expressed in yeast is shown in **Table 1**.

While inserting mammalian cDNA into expression vector by standard molecular techniques of subcloning can be tedious, many mammalian CYP cDNAs are now commercially available in gateway compatible DNA vectors. Gateway compatible vectors contain small segments of DNA, referred to as attP and attB sites, which

CYP gene	Expression vector	Enzymatic assays	Carcinogen activation	Genotoxic assays/ biosensor reporter	References
CYP1A1	pSB229, pRS424 CYP1A1	EROD[1]	BaP-DHD[4]	HR[9], mutation, growth curves	[46, 50, 51]
			AFB1[5]	HR, mutation, growth curves Yfp-Rad51 foci	[46, 50, 51]
			IQ[6]	Growth curves	[51]
CYP1A2	pCS316, pAAH5N	EROD,MROD[2], LND[3]	AFB1	HR, mutation Yfp-Rad51 foci	[43, 50]
			MeIQx[7]	HR	[87]
			IQ	HR	[87]
CYP1A2/ NAT2	pGP100	MROD, SMZ assay	IQ, MeIQx, MeIQ	HR	[87]
CYP1B1	pYES2, pAG24	EROD	AFB1, BaP-DHD		[111]
CYP2A6	pAAH5N	LND			[43]
CYP2B6	pAAH5N, pESC-URA3	LND, 7-ethoxycoumarin-3-carbonitrile deethylation	N-nitrodimethyl amine		[43]
			AFB1	RAD54-GFP	[71]
CYP2C8	pAAH5N	LND			[43]
CYP2C9	pAAH5N	Lauric acid (omega-1)-hydroxylation			[43]
CYP2C18	pAAH5N	LND			[43]
CYP2D6	pAAH5N, pESC-URA3	LND, debrisoquine 4-hydroxylation, ethoxycoumarin-3-carbonitrile deethylation		RAD54-GFP	[71]

CYP gene	Expression vector	Enzymatic assays	Carcinogen activation	Genotoxic assays/ biosensor reporter	References
CYP2E1	pAAH5N	Lauric acid (omega-1)-hydroxylation			[43]
CYP3A4	pAAH5N, pMA34, pESC-URA3	Diclorofenac, testosterone 6β-hydroxylation, ethoxycoumarin-3-carbonitrile deethylation	AFB1	HR, growth curves RAD54-GFP, RNR3-GFP	[67, 71]
			IQ		[67]
			BaP[8]	RAD54-GFP	[71]

[1]*Ethoxyresorufin deethylase (EROD).*
[2]*Methyoxyresorufin demethylase (MROD).*
[3]*Lidocaine N-deethylation (LND).*
[4]*Benzo[a]pyrene 7,8, dihydrodiol (BaP-DHD).*
[5]*Aflatoxin B1 (AFB1).*
[6]*2-Amino-3-methylimidazo-[4,5-f]quinoline (IQ).*
[7]*2-Amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx).*
[8]*Benzo[a]pyrene,*
[9]*homologous recombination (HR).*

Table 1.
Human P450 genes that have been expressed in yeast.

flank the insert and are substrates for site-specific recombinases [44]. CYP cDNAs inserted into donor vectors can then be transferred into recipient yeast expression vectors by mixing the appropriate DNAs with recombinases; these reagents are commercially available and eliminate protocols using restriction enzymes and ligase. Recipient yeast expression vectors include multi-copied vectors as well as inducible and constitutive promoters [44]. An additional mechanism to increase CYP expression is to enhance translation of mRNA; Kozak sequences can be inserted into DNA sequences that encode mRNA upstream untranslated regions (UTR) [45].

2.2 Assays for detecting CYP expression

Enzymatic assays to measure CYP activity have often relied on converting non-fluorescent substrates into fluorescent products or measuring products by high-performance liquid chromatography (HPLC). Fluorescent products can be measured in a 96-well plate on a plate reader. The assay mix involves NADPH or a NADPH-regenerating system, such as glucose dehydrogenase; the pH is critical so the assay mix must be carefully buffered [46]. Microsome preparations of cytochrome P450s from yeast involve lysing cells using glass beads, centrifugation to remove debris, and precipitating microsomes using NaCL and polyethylene glycol [47]. These microsome fractions can be further concentrated by ultracentrifugation and stored at −80°C for extended time periods. Activity measurements are expressed as picomole of product per minute per mg protein; more precise measurements of CYP protein concentration can be obtained by measuring absorbance at a 450nm wavelength after the sample has been exposed to CO.

To optimize mammalian enzyme activity in yeast cells, it is necessary to co-express the CYP, human oxidoreductase (hOR), and cytochrome B (cytB)

oxidoreductase [48]. Because yeasts contain endogenous oxidoreductases [49], the overexpression of the hOR is not a requirement for expression of all CYPs but generally does enhance CYP activity. For example, expression of hOR is required to measure CYP1A1 but not CYP1A2 activity [49, 50]. Other investigators have shown that the insertion of hOR directly in the genome is sufficient to obtain extracts to monitor the activity of CYP1A1 and CYP3A4 [51, 52].

3. Genotoxic assays

To be proven positive, the genotoxic effects must be dose dependent and reproducible. Examples of genotoxic agents include those that directly bind to or modify DNA, induce reactive oxygen species (ROS), and inhibit topoisomerases and other proteins involved in DNA metabolism. These genotoxic agents can cause a multiplicity of DNA insults, including DNA base modifications, DNA adducts, cross-links, and single- and double-strand breaks. Different DNA damage insults can quantitatively result in different biological endpoints. For example, a single double-strand break is sufficient to initiate genome rearrangements and trigger cell cycle arrest [53], while other types of DNA damage, such as particular cross-links and abasic sites, are effectively tolerated by DNA replication bypass pathways (for reviews, [54]). These replication bypass pathways include template switching and error-free polymerase switch mechanisms that may not trigger cell cycle arrest or a DNA damage response [54]. Thus, there is a need for measurements of multiple genotoxic endpoints to accurately assess the biological effect of any genotoxin.

Genotoxic endpoints include direct measurements of DNA damage and DNA adducts, reporter assays that detect transcriptional induction of DNA damage-inducible genes, growth assays for monitoring fitness [55], and plate assays for detecting recombination and mutations. Reporter assays involve yeast strains that contain a DNA damage-inducible promoter linked to a protein tag whose fluorescence or activity can be readily detected. Examples of proteins whose activity can be readily measured include lacZ, encoding β-galactoside, and GUS encoding β-glucuronidase (reviewed in [1]). Signaling assays have been successfully employed for high-throughput analysis using 96-well plate platforms and flow cytometry. The plate assays can elucidate endpoints of genotoxicity, while reporter assays can identify a chemical as a genotoxic assay and establish minimum concentrations in which a chemical may have an effect. Plate assays have been successful in measuring multiple genotoxic endpoints, including mutation [56, 57], homologous recombination [2], retrotransposition [58], and gross chromosomal rearrangements [59]. Plate assays involve inoculating engineered yeast strains on selective media, and after an incubation period, selected colonies can be counted and viability can be measured on nonselective media.

Direct assays to measure DNA strand breaks include chromosomal DNA integrity by pulse-field electrophoresis [60] and by single-cell comet assays [61]. Pulse-field electrophoresis has been successfully used to monitor repair of radiation-induced double-stranded DNA and the integrity of rDNA. Single-cell comet assays involve exposing cells to chemical agents, embedding them in agarose, subjecting them to an electric field, and staining for DNA [61]. Fragmented DNA migrates faster in an electric field, and the fragmented DNA appears as a "tail" [62]. Chemical DNA adducts, such as AFB1-N7-guanine adducts, can be detected using high-performance liquid chromatography, mass spectrometry (LC/MS–MS) after cells have been lysed and DNA has been extracted and acid hydrolyzed [63, 64].

3.1 Reporter assays

Reporter assays with fluorescent readouts are useful in detecting cells that have been exposed to genotoxins that induce DNA damage. Fluorescence can be monitored using 96-well plates, rendering it possible to perform high-throughput analysis. Fluorescent cells can also be imaged using flow cytometry platforms, such as the Amnis Image Stream [65], which can also measure cell type, DNA content, and cell cycle stages. DNA damage reporters include *RAD54*-GFP [66], *RNR3*-GFP [67], and *HUG1*-GFP [68]. These fusions have been widely used because signal-to-noise ratio is very low. *RAD54* is a DNA repair gene that functions in recombinational repair of double-strand breaks; GreenScreen assay (GSA) utilizes the *RAD54*-GFP reporter in high-throughput screens [69]. The RadarScreen assay uses a *RAD54* β-galactosidase reporter construct in which β-galactosidase cleaves the substrate into galactose and luciferin [70]. *HUG1* encodes an inhibitor of ribonucleotide reductase, while *RNR3* encodes the large subunit of ribonucleotide reductase that is specifically induced because of DNA damage [71]. The fluorescent markers can be enhanced using yEGFP, engineered for yeast codon bias. While *RNR3* and *RAD54* promoters have been extensively used, promoters for *PLM2,* encoding a putative transcription factor, and for *DIN7*, encoding a mitochondrial nuclease, have been recently reported to be more inducible for detection of genotoxins [1]. These studies indicate that there are robust reporters with sensitive readouts for screening genotoxins.

Genotoxins that inhibit histone deacetylases, such as Sir2, can be detected using reporters that detect expression of the silent mating-type locus (*HML*). In a strain containing one such fluorescent reporter, the cre recombinase gene was placed within the *HML* locus, while loxP recombination sequences were positioned flanking an intervening sequence that occluded a promoter from transcribing the GFP reporter [72]. Transient expression of cre triggers recombination at the loxP sites and excision of the intervening sequence, thus allowing the promoter to transcribe GFP. While this assay does not directly measure genotoxicity, inhibition of Sir2 can trigger rDNA instability [73].

3.2 Plate assays for detecting recombination, mutation, and microsatellite instabilities

Plate assays that detect mutation and recombination endpoints consist of selections or screens for prototrophic or drug resistance markers. Several genotoxic endpoints can be determined by color phenotypes. For example, Ade^+ colonies are white, while *ade2* and *ade3* colonies are red. Recombination between two nonrevertible *ade2* and *ade3* alleles can be observed by visualizing colony sectors [74]. A similar scheme can also be employed for detecting mutations that affect the *SUP4-o* function in suppressing *ade2* nonsense alleles [75]. By choosing different prototrophic selections for individual assays, combinations of these assays in a single strain can facilitate measurements for multiple genotoxic endpoints, including mutation and recombination. Typically, the strains are diploids. Prototype strains included D7, which allows for measuring multiple number of mutation event [57]. For designing strains to detect DNA damage-associated homologous recombination between heteroalleles or between repeated sequences (ectopic recombination), gene editing and one-step gene replacement [76] rendered it possible to position nonrevertible markers in tandem, on homologs or on nonhomologous chromosomes. For example, *ade2-a* and *ade2-n* alleles can be used for measuring intrachromatid gene conversion and recombination between homologs [74]. By deleting the entire wild-type gene, and positioning overlapping gene fragments at preselected

positions in the yeast genome, frequencies of rare recombinants can be selected. For example, *his3* gene fragments, *his3-Δ3'* and *his3-Δ5'*, can be positioned at predetermined positions in the genome and His^+ recombinants for measuring frequencies of unequal sister chromatid exchange (SCE), translocations, and intrachromatid deletions [77]. Schiestl et al. [2] used a diploid strain that contained a *his3* deletion on one chromosome and a disrupted *HIS3* gene to measure intrachromatid recombination; this strain has also been referred to as the "DEL" assay. The complete deletion of *HIS3*, *his3-Δ200*, also enabled a selection for monitoring Ty1 transposition. Boeke et al. [78] inserted an artificial intron in an inverted orientation within *HIS3* contained within Ty1 so that His^+ cells could only result when retrotransposition of Ty1 occurred. Transposition of *HIS3*, as well as chromosomal rearrangements generated by recombination between *his3* fragments, can be physically characterized by pulse-field gel electrophoresis (**Figure 2**).

A plate assay that detects gross chromosomal rearrangements was devised in haploid strains. This assay involved two drug selection markers, *CAN1* and *URA3*, where the *URA3* gene was inserted near *CAN1* on the right arm of chromosome V at the *HXT13* locus; the right arm of chromosome V is not required for viability [79]. *CAN1* encodes the arginine permease gene and confers sensitivity to the arginine analog, canavanine, while *URA3* confers sensitivity to the drug 5-fluoro-orotic acid (FOA). Since the frequencies of spontaneous Can^R FOA^R is extremely low, 3.5×10^{-10} [79], most Can^R FOA^R selected colonies contain gross chromosomal rearrangements, in which deletions, translocations, or multiple rearrangements have

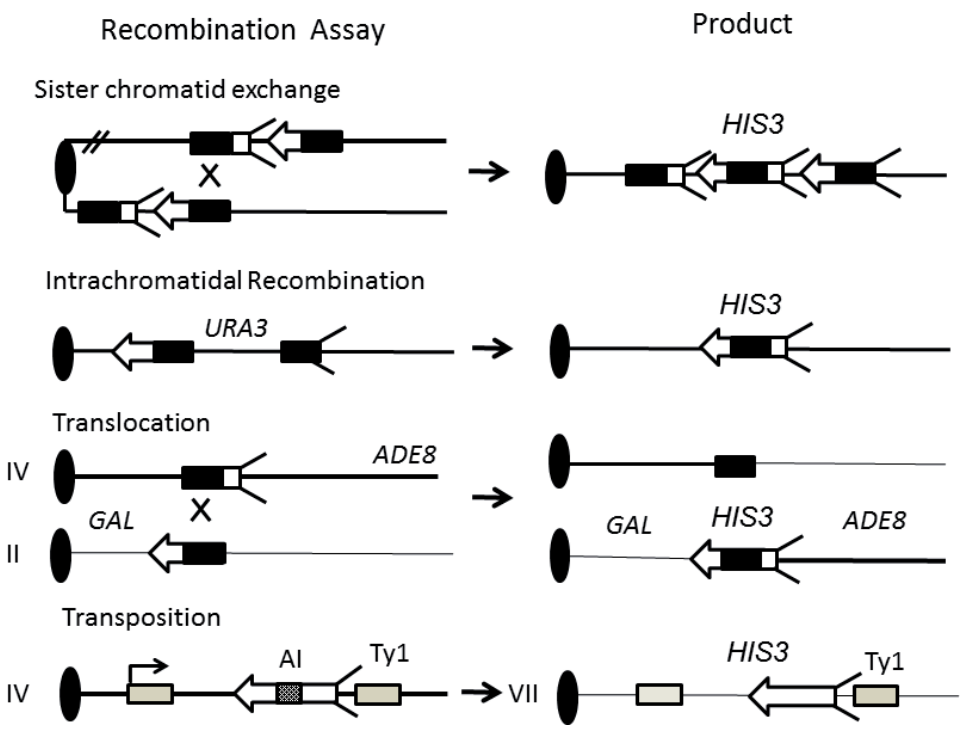

Figure 2.
Recombination assays that are used in detecting DNA damage-associated recombination. The HIS3 *gene is shown with an arrow and feathers. The fragment that lacks the 3' end is shown without the arrow; the fragment that lacks the 5' end is shown without the arrow. The two fragments share approximately 300 bp of homology. The Roman numerals represent different chromosomes. The oval represents the centromere. For simplicity, the left arm of the chromosomes is not shown. In the transposition assay, an artificial intron (AI) is inserted in the* HIS3 *so that it is in the opposite orientation as the* HIS3 *promoter. For* HIS3 *to be expressed, the Ty1 element must first be transcribed, the AI spliced from the mRNA, and the mRNA reverse transcribed and integrated into the chromosome.*

occurred that conferred resistance to both drugs. However, because drug sensitivity is dominant, Can^R FOA^R recombinants are detected in haploid strains.

By combining different gene fragments and alleles, as well as drug-resistant markers, multiple genotoxic endpoints, including heteroallelic recombination, unequal SCE, translocations, and mutation, can be measured within a single strain. As an example, Fasullo et al. [64] designed a haploid strain useful in measuring frequencies of DNA damage-associated mutations and unequal SCE after exposure to AFB1. A useful diploid strain was also engineered for measuring frequencies of DNA damage-associated homolog recombination between heteroalleles and ectopic recombination between gene fragments on nonhomologous chromosomes [64]. While these plate assays can elucidate genotoxic endpoints, their noise-to-signal ratio can vary, depending on the frequencies of spontaneous events. While frequencies of spontaneous mutations at *CAN1* are relatively low, 10^{-6}, the frequencies of spontaneous recombinants can vary from 10^{-4} to 10^{-10} [79]. Higher frequencies of spontaneous recombination are generally associated with intrachromosomal events, while lower frequencies of spontaneous recombination are associated with ectopic recombination between gene fragments on nonhomologous chromosomes. The lower the spontaneous frequency infers the higher the signal-to-noise ratio; thus, DNA damage-associated recombinants may be identified at exposures to lower concentrations of genotoxins.

While there are a multitude of plate assays for detecting nuclear genotoxic stress, there are fewer assays for detecting mitochondrial genotoxic stress. In part this is due to few auxotrophic markers, the high copy number (50–100) of mitochondrial DNA, and random segregation of mitochondria in mitosis [80]. Nonetheless, mitochondrial deficient yeast can be detected by the petite colony phenotype and the color phenotype of Ade^- mutants that appear pink or white in contrast to red on YPD media that is limiting in adenine [81]. In addition, Sia et al. [82] constructed a mitochondrial reporter gene arg8(m). This reporter has poly(AT) or poly(GT) out-of-frame insertions within the coding sequence so that Arg^+ prototrophs can be selected resulting from microsatellite instability.

While the plate and reporter assays are useful for detecting genotoxins and elucidating their mechanisms, yeast lacks many metabolic activities found in metabolically competent mammalian cells. Some protocols to activate carcinogens use rat S9 fractions, which may produce more metabolites than human CYPs, [83–85]. To mitigate this deficiency, human CYPs have been introduced into the strains for both plate assays and reporter assays. For example, Bui et al. [71] expressed CYP1A2, CYP2C9, CYP3A4, and CYP2D6 in a strain that monitors RAD54-GFP. Sengstag et al. [50] and Fasullo et al. [64, 67] have expressed CYP1A1, CYP1A2, and CYP3A4 in strains that monitor translocations, mutations, and unequal SCE. Guo et al. [86] have introduced CYP1A2 into multiple yeast mutants to determine AFB1 resistance. Paladino et al. [87] have expressed CYP1A2 and NAT2 to activate a variety of heterocyclic aromatic amine in strains to measure homology-directed translocations. Both CYP-containing reporter strains and plate assay strains expand the repertoire of chemicals that can be tested by high-throughput analysis.

4. Chemicals that test positive in the yeast strains

Overall, thousands of chemicals have been tested using either one or both plate and reporter-based assays [1]. Van Gompel et al. [69] report on the screening of 2698 proprietary compounds and pharmaceuticals using the GreenScreen assay; of these compounds, approximately 7% of those 164 that test positive are also positive in the Ames assays, demonstrating that agents that test positive represent

overlapping groups. Screens of industrial, environmental, and food carcinogens have used multitude tester strains, including the "DEL" and transposition assays [88]. Chemical agents include those that directly inflict DNA damage, induce ROS, inhibit DNA metabolic function, and alter histone modification. Metallic nanoparticles also test positive in several assays although their mechanism of action has yet to be determined [89]. Whereas almost all chemicals that test positive in plate assays will also test positive in reporter assays, the converse is not necessarily true. These results demonstrate that several reporter assays are capable of high-throughput screening and can identify multiple compounds that test positive in additional genotoxic assays.

Several agents that cause direct DNA damage, such as base pair damage, cross-links, DNA adducts, or DNA strand breaks, test positive in reporter assays and may test positive in one or more of the plate assays [90]. For example, alkylating agents, such as methyl methane sulfonate (MMS), increase frequencies of mutations, recombination, gross chromosomal rearrangements (GCRs), and retrotransposition. Interestingly, alkylating agents also test positive in enhancing expression of the silent mating-type locus *HML* [91]. Other types of alkylating agents, such as 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and ethyl methane sulfonate (EMS), test positive in reporter and mutation assays; however, frequencies of DNA damage-associated sister chromatid exchange events are only modestly increased [58, 64, 67]. Cross-linking agents, such as cis-platinum and other UV-mimetic agents, also tend to be positive in a broad range of reporter assays, including those for retrotransposition and homologous recombination [90]. Finally, radiomimetic agents that cause strand breaks, such as zeocin and bleomycin, test positive in many reporter assays as well as assays for gross chromosomal rearrangements and translocations [59]. These studies demonstrate that while a subset of alkylating agents manifest broad genotoxicity, frequencies of DNA damage associated with GCRs and translocations manifest the highest increase after exposure to agents that cause double-strand breaks.

Chemical agents that inhibit DNA metabolic and repair functions are often genotoxic. These include camptothecin, which inhibits topoisomerase I and causes single-strand breaks and replication fork collapse, and hydroxyurea, which blocks DNA replication by inhibiting ribonucleotide reductase and thus depleting deoxynucleotides [92, 93]. Other metabolic inhibitors include those that inhibit dihydrofolate reductase, and result in uracil incorporation also tests positive in a broad range of plate assays, including those for sister chromatid recombination, heteroallelic recombination, and translocations. Cd^{2+} exposure inhibits mismatch repair [94] and is also genotoxic [95]. These studies indicate that genotoxins include chemicals that may directly inhibit critical enzymes in DNA metabolism.

While chemicals are individually screened in many plate and reporter assays, combination of chemicals can also enhance DNA damage or enhance mutagenesis. An example includes intercalating agents, such as acridine and bleomycin; the insertion of acridine in the DNA helix facilitates bleomycin access to the minor groove and subsequent strand breakage [96]. In addition, by inhibiting mismatch repair, Cd^{2+} exposure facilitates the mutagenesis by alkylating agents [94]. These studies indicate that combinations of genotoxins can accelerate genome instability.

Mitochondria are particularly prone to DNA intercalating agents, and agents that cause oxidative damage, and reduce or cause imbalance to deoxynucleotide pools [97]. ROS-associated damage in the mitochondrial genome, associated with oxidative phosphorylation, is not repaired by nucleotide excision repair (NER) but by base excision repair (BER) [1]. In addition, mitochondrial DNA is circular and therefore is more prone to DNA intercalating agents that can cause topological stress, such as ethidium bromide and acridine compounds [98]. Several fluorescent

CYP allele	Amino acid substitution	Enzyme assay	Disease association	Genotoxic endpoints	Reference
*CYP1A1*2C*	1462V (near heme binding site)	EROD[1]	Lung, prostate and breast cancer	Rad51 foci, growth curves, HR[4]	[51]
*CYP1A1*4*	T461N (near heme binding site)	EROD	Endometrial and lung cancer	Rad51 foci, growth curves, HR	[51]
*CYP1A2*5*	C406Y	MROD[2]	ND	Rad51 foci, growth curves, HR	[63]
*CYP1A2*3*	D348N	MROD	ND	Rad51 foci, growth curves, HR	[63]
*CYP1A2*4*	I386F	MROD	ND	Rad51 foci, growth curves, HR	[63]
*CYP1B1*7*	R48G; A119S; L432V; A443G	BaP-DHD epoxidation[3]	L432V has an increased risk for prostate and lung	NT	[110]
*CYP1B1*12*	G61E	BaP-DHD epoxidation	Glaucoma	NT	[110]
*CYP1B1*18*	G365W	BaP-DHD epoxidation	Glaucoma	NT	[110]
*CYP1B1*23*	P437L	BaP-DHD epoxidation	Glaucoma	NT	[110]
CYP2E1		Benzene hydroxylation		NT	[111, 112]
*CYP2E1*2*	**R76H**	Benzene hydroxylation	Bladder cancer (reduced risk in Asian population)	NT	[111, 112]
*CYP2E1*3*	V389I	Benzene hydroxylation	Bladder cancer (reduced risk in Asian population)	NT	[111, 112]
*CYP2E1*4*	V179I	Benzene hydroxylation	Bladder cancer	NT	[111, 112]

[1]*Ethoxyresorufin deethylase (EROD).*
[2]*Methyoxyresorufin demethylase (MROD).*
[3]*Benzo[a]pyrene 7,8, dihydrodiol (BaP-DHD).*
[4]*Homologous recombination (HR).*
ND = not determined, NT = not tested.

Table 2.
Cytochrome P450 polymorphisms expressed in yeast.

dyes can also induce mitochondrial DNA damage [1]. Replication of mitochondrial DNA depends on a single polymerase, DNA polymerase γ [99]. Therefore, chemicals that inhibit mitochondrial DNA polymerase, such as dideoxynucleoside antiretrovirals, are often genotoxic [100]. Thus, yeast screens that detect mitochondrial DNA damage are useful in screening off-target effects on antiretroviral agents.

While many carcinogens are directly genotoxic, others require metabolic activation. The list of CYPs expressed in yeast and chemical agents that are activated are listed in **Table 2**. The agents tested include polyaromatic hydrocarbons (BaP-DHD), mycotoxins (AFB1), and heterocyclic aromatic amines (2-amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx), 2-amino-3, 4-dimethylimidazo-[4,5-f] quinoline (MeIQ), and 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ)). Bui et al. [71] introduced human CYPs into strains to measure induction of GFP using the reporter *RAD54*-GFP. Li et al. [52] used a sensitive fluorimetric assay to measure inhibition of secreted dextranase; the assay consists of strains expressing *Lipomyces kononenkoae* amylase, CYP3A4, and hOR [52]. The amylase-expressing strain detected AFB1 at 2 ng/ml and the T-2 mycotoxin [52].

Activation of these compounds has also been determined by measuring DNA recombination and mutation; DNA adducts have been detected after AFB1 and BaP-DHD exposure. Frequencies of mutations and recombination may be differentially elevated by CYP-activated genotoxins. For example, CYP1A1 and CYP1A2 activation of AFB1 in yeast results in a 20–50-fold increase in the stimulation of recombination but only a fivefold increase in mutation frequency [50]. However, CYP1A1-mediated activation of BaP-DHD results in a higher activation of mutation but somewhat diminished activation of recombination [50]. Because the background frequency is so low, the CYP1A2-expressing strains containing the translocation assay have been particularly useful in detecting the DNA damage-associated recombinants [50].

5. Yeast mutants that exhibit enhanced phenotypes after genotoxin exposure

Various gene mutations can increase the signal-to-noise ratio. Typically, these mutations are encoded in cells lacking cell wall components, nucleotide or base excision repair genes, and xenobiotic transporters. Strains that lack cell wall components and xenobiotic transporters include *pdr5*, *snq2*, *cwp1*, *cwp2* [1]. Strains that lack NER and BER genes include those mutated in *rad1* and *mag1*, respectively. Several strains also contain deletions in *yap1* [101], a gene that encodes a transcription factor that confers transcriptional induction among antioxidant genes, such as *TRX2*, and is required for H_2O_2 and Cd resistance [102]. Several strains have been designed so that multiple genes are mutated to enhance the genotoxic signal. Wei et al. [103] have used a septuplet deletion mutant (*snq2*, *prd5*, *cwp1*, *cwp2*, *yap1*, *rad1*, *mag1*) in combination with an integrated HUG1-yEGFP reporter as a very sensitive detection for multiple chemicals. Deletion of NER genes has been successful in enhancing DNA damage-associated recombination after exposure to BaP-DHD and AFB1 [104].

While deleting DNA repair genes may enhance signal-to-noise ratios for reporter assays and some recombination and mutation assays, particular DNA repair defects may decrease frequencies of DNA damage-associated recombination in particular plate assays. For example, blocking nonhomologous end joining (NHEJ) may increase homologous recombination initiated by double-strand breaks in haploid strains, while decreasing DSB-associated translocations [105]; the likely explanation is that competing DNA repair pathways for recombination are differentially favored for homologous vs. NHEJ. *Rad1* mutations may confer lower DNA damage-associated recombination in assays, such as the "DEL" assay [106]. The *rad1* mutants are defective in the Rad1/Rad10 nuclease; this nuclease cleaves 3′ blocked termini and is important in single-strand annealing mechanisms. However, other *rad* mutants that are deficient in NER, such as *rad4*, may be suitable for observing both enhanced DNA damage-associated recombination and mutations [67].

One strategy has been to use DNA repair mutants that are knocked out in multiple DNA repair pathways to assess the genotoxicity of chemicals. For example, *rad4 rad51* double mutants, which are deficient in both NER and in recombinational repair, are synergistically more sensitive to UV and many chemical UV-mimetic agents. By introducing CYP genes into the *rad4rad51*, the strain also becomes synergistically more sensitive to AFB1 as well as extremely sensitive to other PAHs and HAAs [51, 67].

Mechanistic insights into how genotoxic agents stimulate chromosomal instability are also gained from studies of checkpoint genes. For example, deleting the *RAD9*-mediated checkpoint which triggers G2 arrest confers higher levels of DNA damage-associated recombination (translocations) after cells are exposed to X-rays and radiomimetic agents that cause breaks such as MMS and bleomycin. Enhanced translocation frequencies are also observed after *rad9* cells are exposed to camptothecin, an inhibitor of topoisomerase I. On the other hand, *rad9* deletion does not confer higher levels of recombination associated with agents, such as 4-nitroquinoline oxide (4-NQO), that cause bulky DNA damage [107]. These observations suggest that agents that stimulate DNA break formation may be identified if they enhance recombination in *rad9* mutants.

6. Phenotyping CYP polymorphisms in budding yeast

The CYP genes are highly polymorphic, and particular polymorphisms have been identified as risk factors for cancer [13, 22, 108] and glaucoma [109]. While yeast strains are useful in elucidating the genotoxicity of P450-activated carcinogens, yeast strains are also useful in characterizing human CYP polymorphisms. CYP1A1, CYP1A2, CYP1B1, and CYP2E1 polymorphisms have been studied in yeast [51, 63, 110–112]. The polymorphisms can be characterized in a number of ways: (1) substrate specificity, (2) activity with a defined substrate, (3) genotoxic endpoints, and (4) DNA adducts. For example, CYP1A2 polymorphisms have different affinities for heterocyclic aromatic amines; these polymorphisms have been also characterized by their ability to bioactivate aflatoxin B1. Activity assays have been performed for polymorphisms in CYP2E1, CYP1B1, CYP1A1, and CYP1A2 [51, 63, 110, 111]. In general activity assays agree with those performed when assays are performed in other model systems, such as *E. coli* [113].

Several CYP1A1 polymorphisms are present in a significant percentage of the population and may be risk factors for lung and breast cancer. For example, CYP1A1 I462V and CYP1A1 T461N have been correlated to have higher incidence of lung, breast, and endometrial cancer [114, 115]. A plausible hypothesis is that CYP1A1 I462V and CYP1A1 T461N are more active in converting breast- and lung-associated carcinogens into genotoxins. However, another model suggests that CYP1A1 is protective, since CYP1A1 knockout mice actually have a higher incidence of carcinogen-associated cancer [10]. Freedland et al. [51] measured multiple genotoxic endpoints in yeast strains expressing CYP1A1 I462V after exposure to multiple carcinogens and interestingly found a reduced level of bioactivation. This is consistent with a model that CYP1A1 may actually be protective and compete with other CYPs that convert carcinogens into active genotoxins [10].

7. Implications for higher eukaryotes

The ability to perform high-throughput screening to identify genotoxins using yeast strains containing sensitive reporter facilitates the identification of chemicals that merit more detailed and expensive studies. While yeast reporter strains can

be useful for high-throughput identification of genotoxins, yeast plate assays and genetics can elucidate mechanisms. Genotoxins that stimulate recombination and retrotransposition in yeast are likely to stimulate genetic instability in higher eukaryotes. Indeed, many recombinagens that have tested positive in yeast also test positive in higher eukaryotes. An excellent example is AFB1, which is also a recombinagen in human cell lines [116].

8. Conclusions and future directions

Yeast assays for detecting genotoxins and identifying genotoxic mechanisms are urgently needed to screen a multitude of industrial chemicals, pesticides, and pharmaceuticals. These assays have already been successful in screening thousands of chemicals, aiding in our understanding of genotoxic mechanisms. These assays have been further empowered by the technology to introduce human phase I and phase II metabolism in yeast cells. While the reporter assays enable high-throughput studies for rapid identification of genotoxins, the multitude of plate assays enables mechanistic studies to elucidate genotoxic mechanisms. The future challenge is to combine many of the reporters and plate assays so that both the screening and the mechanistic studies can be expedited.

Currently, the mechanisms of many chemical agents, which increase cancer risk, are unknown. Of particular interests are many small-molecule toxicants present in industrial workplace or which are extensively used in agriculture. How exposure to mixtures of these chemicals increases genotoxicity will be important in assessing risk factors to human health.

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Conflict of interest

The author declares no conflict of interest.

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Chapter 2

Random Mutagenesis of Filamentous Fungi Strains for High-Yield Production of Secondary Metabolites: The Role of Polyamines

Alexander A. Zhgun

Abstract

A filamentous fungus (also called molds or moldy fungus) is a taxonomically diverse organism from phylum *Zygomycota* and *Ascomycota* with filamentous hyphae and has the ability to produce airborne spores or conidia. Currently, more than 70,000 molds are known, and some of them contain unique and unusual biochemical pathways. A number of products from such pathways, especially, the secondary metabolite (SM) pathways are used as important pharmaceuticals, including antibiotics, statins, and immunodepresants. Under different conditions, the individual species can produce more than 100 SM. The strain improvement programs lead to high yielding in target SM and significant reduction of spin-off products. The main tool for the strain improvement of filamentous fungi is random mutagenesis and screening. The majority of industrial overproducing SM strains were developed with the help of such technique over the past 50–70 years; the yield of the target SM increased by 100- to 1000-fold or more. Moreover, most of the strains have reached their technological limit of improvement. A new round of mutagenesis has not increased overproduction. Recently, it was shown that that the addition of exogenous polyamines may increase the production of such improved strains of filamentous fungi. The possible molecular mechanism of this phenomenon and its biotechnological applications are discussed.

Keywords: filamentous fungi, random mutagenesis and screening, strain improvement, secondary metabolites, polyamines

1. Introduction

Improved strains of filamentous fungi are widely used in the biotechnology industry for recycling of secondary raw materials [1–3] as biosorbents [4], in fermentation of cheese [5], wine [6, 7], and other food products [8, 9], as well as for the production of enzymes [10–13], organic acids [14, 15], secondary metabolites (SMs) [16, 17], or for steroid transformation [18, 19]. There are four main tools for fungal strain improvement: (1) sexual crossing [20, 21], (2) somatic crossing (including parasexual recombination [22]), (3) random mutagenesis by physical or/and chemical

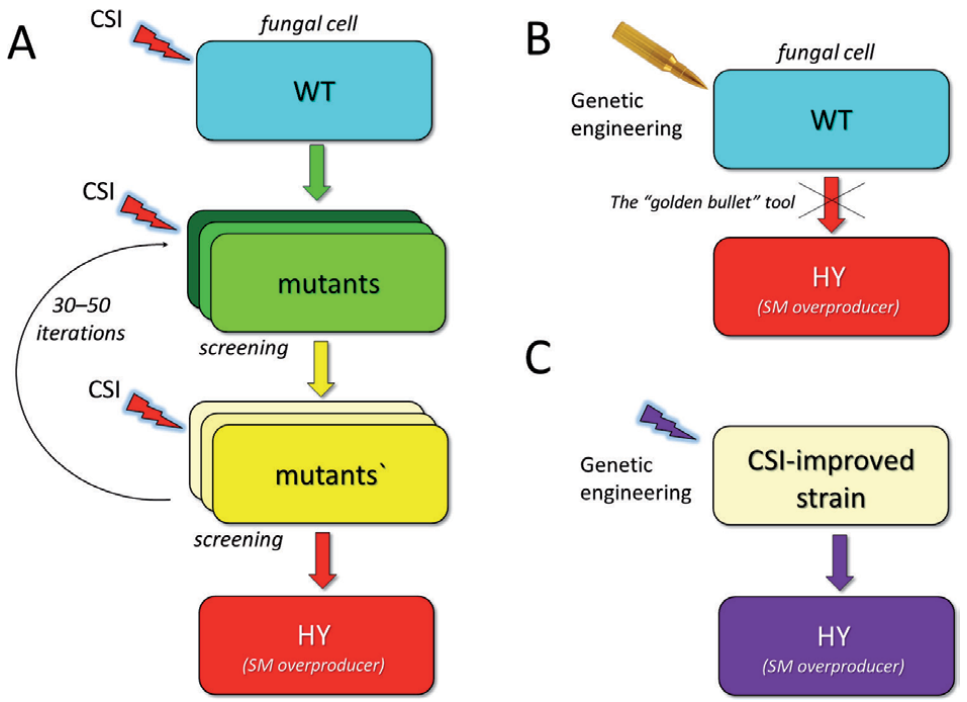

Figure 1.
The improving of filamentous fungi strains for SM production. (A) CSI programs for fungal strain improvement. (B) The WT strain improvement by the "golden bullet" tool from genetic engineering. (C) The combination of SCI and genetic engineering approaches for the developing of novel HY strain. SM: secondary metabolite, CSI: classical strain improvement, WT: wild type, and HY: high yielding.

mutagens and screening [23, 24], and (4) genetic engineering [25–27]. These methods can be applied separately or in various combinations [28]. The first three tools are referred to as classical strain improvement (CSI) methods and have been used in strain improvement programs for filamentous fungi for SM production since the 1950s of the twentieth century (**Figure 1A**). The majority of industrial producers of secondary metabolites in fungi were obtained precisely with the use of CSI [25]. The powerful genetic engineering approach has been available since the end of the twentieth century for targeting the particular genetic determinant to introduce novel properties into an organism [28]. Since the improvement of filamentous fungi strains for SM production is a complex and multistage program that radically changes numerous processes, there is no "golden bullet," any single unique genetic change to produce high yielding (HY) strain from the wild type (WT) strain [29] (**Figure 1B**). However, introducing of novel targeted features into already improved strains enables to create SM-overproducing strains [30]. For instance, the introduction of the compactin pathway from the *Penicillium citrinum*, as well as CYP105AS1 (from *Amycolatopsis orientalis*, for pravastatin hydroxylation) into the β-lactam-negative *P. chrysogenum* DS50662 strain, yielded more than 6 g/L of pravastatin [25]. This seems to be due to the interaction of different tools that are available to improve strains (**Figure 1C**).

2. CSI for SM production in filamentous fungi

For the majority of industrially important filamentous fungi (except members of genera *Aspergillus*, *Claviceps*, and *Emericellopsis*), the sexual breeding is not available [28]. From the other side, a number of these organisms produce haploid

conidia, which provide the ideal material for mutagenic treatment: in the absence of a complementary set of genes, mutations will be easily detected using suitable screening and the stability of the mutant will generally be good [28]. Filamentous fungi turned out to be surprisingly tolerant to strong mutagenic effects, retaining their strength and productivity even after radical rearrangements of their chromosomes [31, 32]. In this case, the main tool of CSI for SM production in filamentous fungi is random mutagenesis mutagens and screening [33].

2.1 The overproduction of target SM in filamentous fungi

The individual species of filamentous fungi under different external and internal signals are able to produce up to 100–150 or more different SMs [34–36]. This is achieved due to the presence in the genomes of these organisms of 30–80 clusters of genes, responsible for various biosynthetic pathways of SMs, so-called biosynthetic gene clusters (BGCs) [37, 38], and by the fine tuning in the regulation of their expression [39, 40]. Currently, more than 20,000 SMs are known to be produced from more than 1000 characterized gene clusters of filamentous fungi [35, 36]. Normally, gene clusters are "silent," the expression level of BGCs is extremely low, and there is practically no biosynthesis of any SMs (**Figure 2A**). For the biosynthesis of particular SM, the corresponding BGC must be "awakened" by some specific signal. For instance, the environmental signal 1 is resulted in the biosynthesis of SM1 (**Figure 2A**). A number of possible SMs, which can be produced in a particular organism after the "awakening" of corresponding BGCs, constitute its biosynthetic capacity for SMs (**Figure 2A**). In order to become an industrial producer, the fungus strain must increase the production the target SM 100- to 1000-fold or more (**Figure 2B**). It is also necessary that under favorable conditions (usually, these are fermentation conditions), the content of spin-off products would be extremely small (**Figure 2B**).

2.2 The molecular mechanisms of SM overproduction in filamentous fungi

An increase in the production of the target SM by 100- to 1000-fold and the elimination of spin-off products under the fermentation conditions in the improved fungal strains (**Figure 2B**) are associated with two main molecular events, the upregulation of genes from target BGC and the knockout of genes from alternative BGC [27, 33, 41, 42]. Since the expression of BGC genes is controlled by the pathway-specific regulation [27, 43, 44], global regulation [45, 46], and global regulation of SM [47–50], the SCI programs are accompanied by changes in such controls. For instance, during CGI program for penicillin G (PenG) production in HY strain (DS17690) two main events occurred, the shift in global regulation of secondary metabolism by introducing mutations in LaeA and VelA and mutations in key enzymes for spin-off SM [33]. That enabled to escape control from the global regulation of SM and involve more than one gene copy of BGC for PenG.

Usually, an increase in the gene dose (introducing several BGC copies for target SM) does not lead to an increase in gene expression. For instance, in the another PenG-overproducing strain (P2niaD18) the enhanced penicillin titer does not strictly depend on the copy number of the cluster [51]. This phenomenon occurs due to the control from the global regulation of SM, which brings only one cluster to work, the rest are silent [35, 52, 53]. Since there are 8 BGC copies for PenG in DS17690 strain, the escape of global regulation resulted in the significant increase in the yield of the target SM [33]. However, the shift in global regulation of SM could also significantly upregulate the expression from alternative BGC [54, 55]. From this point of view, it becomes clear why the CSI program for DS17690 strain

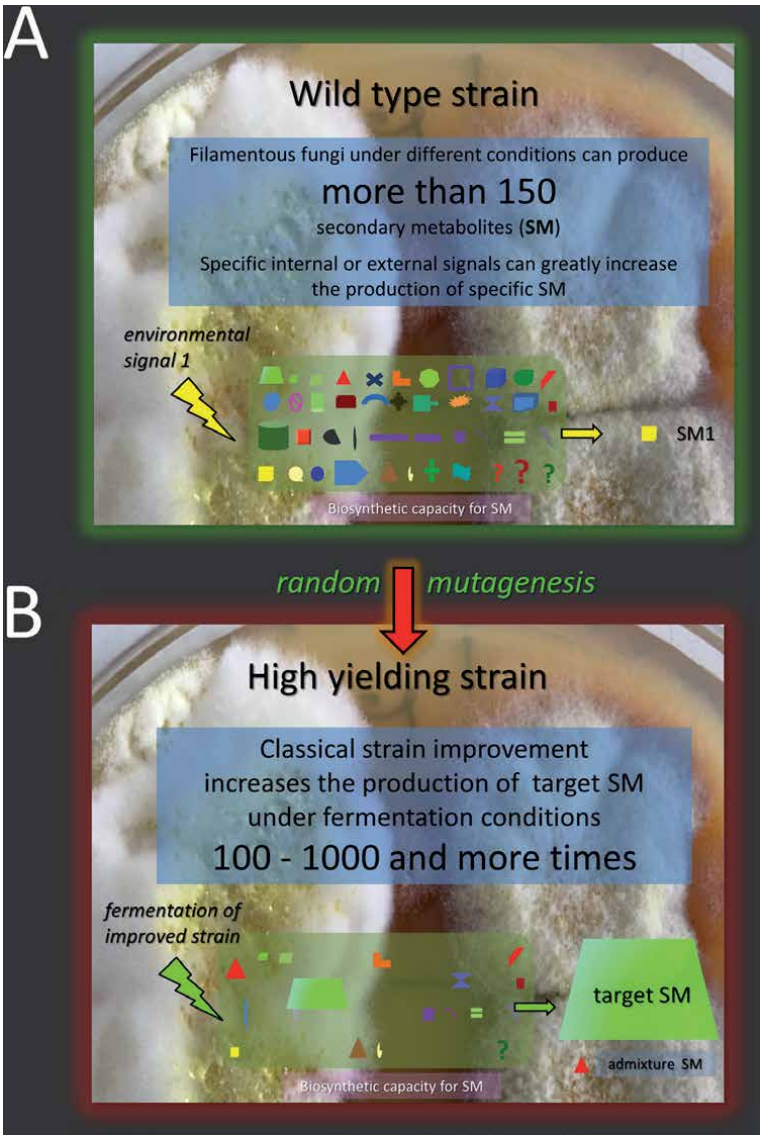

Figure 2.
The shift in biosynthetic capacity for SM production after CSI programs of filamentous fungi. (A) The response of WT strain to the environmental signal 1 and production of SM1. (B) The overproduction of target SM in the HY strain under fermentation conditions. SM: secondary metabolite, CSI: classical strain improvement, WT: wild type, and HY: high yielding.

changes in global regulation of SM also accompanied by mutations in central enzymes for spin-off SM [33]. The shift in global regulation of SM not only took out of control additional BGC copies for PenG, but also – BGC for spin-off SM. Since the screening went against spin-off SM, variants with mutations in central enzymes of alternative BGC were selected [33]. It was demonstrated, that delections in such central enzymes (NRPS or PKS megasynthases) lead by a still unknown mechanism to the silencing of all genes from the corresponding gene cluster [56].

Thus, if the improving of filamentous fungi strains for SM production led to duplication of target BGC, the simultaneous changes both in the system of global regulation of SM and at the level of alternative BGC expression are required [33]. However, in many improved strains, industrial producers of SMs, spin-off products are still formed during fermentation [57, 58]. These impurities are intermediates of the

target SM biosynthesis; their amount depends on numerous fermentation conditions [58]. For instance, the cephalosporin C (CPC) yield after fermentation of improved *Acremonium chrysogenum* strains often contaminated with deacetylcephalosporin C (DAC) [57]. DAC is immediate precursor of CPC in the biosynthetic pathway. The conversion from DAC to CPC is catalyzed by deacetylcephalosporin-C acetyltransferase enzyme (CefG; EC 2.3.1.175) by, occurs in the cytoplasm [59] and is utilizes one molecule of cytoplasmic acetyl-CoA per reaction. In HY strains the CPC production increased 200- to 300-fold and the expression from BGC for CPC (*cef* genes) upregulated 20- to 300-fold [41]. In this case the acetyl-CoA content may be depleted in some HY strains [57, 58]. From the other side the screening during CSI programs went the same way against DAC admixture, events that reduce CPC/DAC ratio were selected. In the *A. chrysogenum* HY strain RNCM 408D [60], the CPC/DAC does not exceed 10–15% [61]. Thus, to increase in SM production is accompanied not only by changes in the expression and regulation of BGCs, but by reprogramming the whole organism, starting with changes in the primary metabolism (for the needs of target SM biosynthesis), ending with changes in the transport and assimilation of nutrients, the ability to assimilate oxygen, adaptation to fermentation conditions, and much more [42]. That is why the improvement of the filamentous fungi strain is a multi-step process, involving alterations in many spheres of the strain's vital activity, and there is no "golden bullet," no one cardinal event that converts WT strain to HY (**Figure 1**).

2.3 The technological limit of CSI of filamentous fungi for SM production

Filamentous fungi are a good facility for the improving of SM production by random mutagenesis and screening [2, 28, 62, 63]. Among the most popular mutagens used for fungal strain improvement are DNA alkylating NTG (N′-methyl-N′-nitro-N′-nitrosoguanidine) which typically produces a variety of point mutations and UV irradiation at 254 nm, which causes the formation of pyrimide dimers leading to point mutations and deletions [28]. In general, the CSI program for SM production in filamentous fungi looks as shown in **Figure 3**. The WT strain produce target SM in most cases at a low level, usually it does not exceed 30–50 μg/ml of fermentation medium [27, 41]. On order to convert WT to HY strain a number of independent events, involving BGCs regulation, changes in primary metabolism, strain physiology and so on, must occur. Moreover, all these events do not have to happen simultaneously. There are a number of ways in which the production of target SM gains added benefit. The first round of mutagenesis against WT strain results in a series of mutants, some of them have shift in the production of target SM (**Figure 3**). The majority of alterations lead to decrease or lack of the production change, but some mutants may show the increase in SM production. They are used as origins for the next mutagenesis round, followed by the next stage of screening. For example, on the A′ round of mutagenesis, the production level of SM was increased by A%, on the B′ round of mutagenesis, the production level of SM was increased by B% (**Figure 3**). Thus the CSI gradually leads to the emergence of a whole set of changes leading to an increase in the production. However, along with beneficial changes that increase the production of the target SM, reduce the amount of spin-off products, and others, numerous side changes begin to accumulate in the fungal strain. They can appear in a slow growth on agar and liquid media [27, 64, 65], a decrease in stress resistance [66], reduction in the conidia formation [64] and many other properties, expressed in a decrease in the overall viability of the strain [66, 67]. Finally, the stage comes when the next mutagenic effect no longer leads to further strain improvement. This is the technological limit of CSI method, it comes for each improvement program for a particular strain of filamentous fungus and is usually found at the 10–50th round of mutagenesis [60, 68].

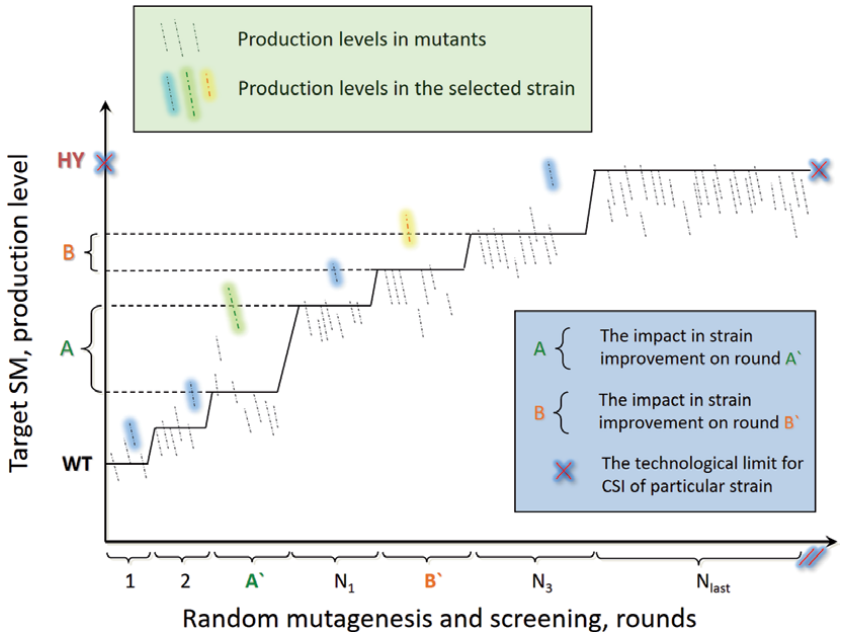

Figure 3.
Random mutagenesis and screening for the improving of filamentous fungi strains for SM production. SM: secondary metabolite, CSI: classical strain improvement, WT: wild type, and HY: high yielding.

3. Role of polyamines in filamentous fungi HY strains (after SCI for SM production)

Aliphatic polyamines (PAs) such as putrescine, spermidine, and spermine are widespread in nature; they are present in all living organisms and are also present in viral particles [69]. Despite the fact that these compounds have long been known as components of biological systems, there is still no clear understanding of their role in various bioprocesses [70]. The most studied functions of PAs are associated with stimulating the growth of microorganisms, increasing membrane stability, interacting with nucleic acids, and regulating the level of heterochromatin in the cell [71–74]. The roles of PAs in fungi cell have also been discussed [71, 75]. The main topics correspond to stress resistance [76], phytopathogenicity [77] and fungal development, including sporulation, growth and other stages of lifecycle [78, 79]. There is tight control of polyamine homeostasis in the cell [80]. For a particular organism, there is a certain content of PAs. For this, there are both biosynthetic and catabolic enzymes of polyamines (**Figure 4**), moreover, the amount of key biosynthetic enzymes, such as ornithine decarboxylase (ODC), or S-adenosylmethionine decarboxylase (AdoMetDC) is regulated at the levels of transcription, translation and turnover rate (half-life) [80, 81].

3.1 Influence of PAs on SM production in improved strains

Recently it was demonstrated, aliphatic PAs, such as 1,3-Diaminopropane (DAP) or spermidine (Spd) may increase the production of target SMs in HY fungi strains [82]. The production level of PenG in *P. chrysogenum* increased by 10–15% [82], the CPC production in *A. chrysogenum* HY strain increased by 10–15% [83] and the production of lovastatin (LOV) by *Aspergillus terreus* HY strain at the particular timepoints of fermentation increased by 20–45% [84]. The addition of 5 mM PAs

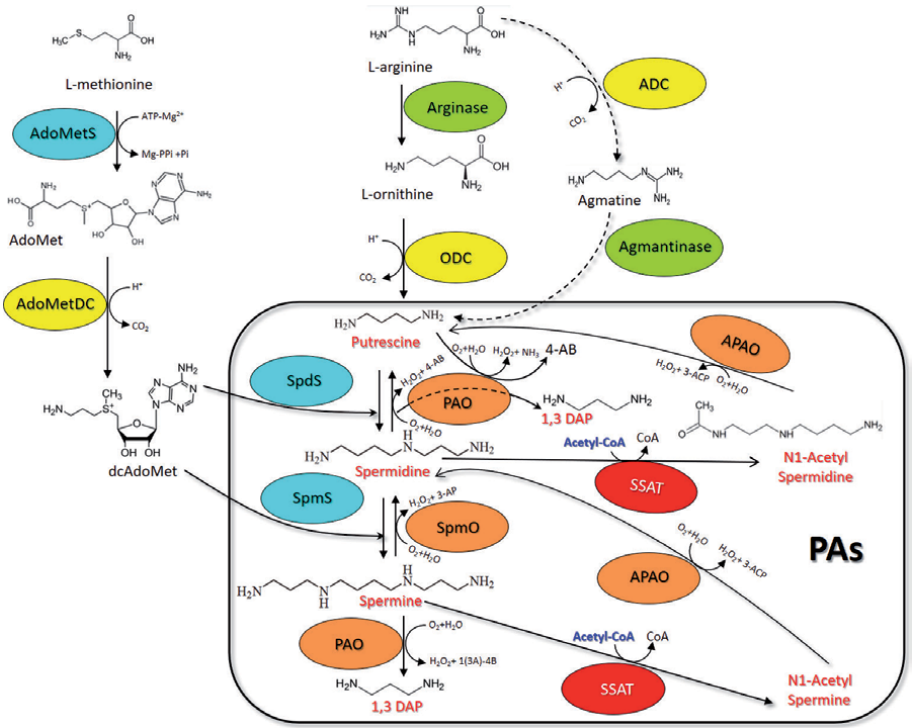

Figure 4.
The metabolism of polyamines in filamentous fungi. PAs: polyamines, ODC: ornithine decarboxylase, ADC: arginine decarboxylase, AdoMetS: S-adenosylmethionine synthetase, AdoMetDC: S-adenosylmethionine decarboxylase, SpdS: spermidine synthase, SpmS: spermine synthase, PAO: polyamine oxidase, SpmO: spermine oxidase, SSAT: spermidine/spermine-N1-acetyltransferase, APAO: N-acetylpolyamine oxidase, DFMO: α-difluoromethylornithine, APA: 1-aminooxy-3-aminopropane, DFMA: α-difluoromethylarginine, and AO-Agm: 1-aminooxy-3-guanidinopropane.

to agar media increased the survival of HY strains, as demonstrated by the drop and dilution assay [83, 84]. The PAs addition during the fermentation of improved strains also led to upregulation of corresponding BGCs (*pen*, *cef* and *lov* genes) [82–84]. This is important because *A. chrysogenum* and *A. terreus* HY strains have reached their technological limit after CSI programs [60, 68] and the possibility of further increasing the production of valuable pharmacologically significant substances as a result of the addition of relatively cheap PAs may be significant in the biotechnology industry.

3.2 Possible mechanisms of influencing of exogenous PAs on SM production

The addition of exogenous PAs also accompanied by the *laeA* upregulation in all studied improved strains [82–84]. LaeA is global regulator of SM in filamentous fungi [50]. It is S-adenosylmethionine (SAMe)-dependent histone methylate, which acts epigenetically, through the chromatin remodeling [85]. Since the biosynthesis of PAs and the work of LaeA require the same substrate, SAMe, the addition of exogenous PAs can lead to a shift in the global regulation of the studied HY strains. It is also known that in all these strains, *P. chrysogenum* Wis 54-1255, *A. chrysogenum* RNCM 408D and *A. terreus* No. 44-62, the only one copy of corresponding BGC is present, one copy of *pen* genes [86], one copy of "early" and "late" *cef* genes [32, 41] and one copy on *lov* genes [84] respectively. In this regard, the CSI programs for these strains could follow a rather different pathway than *P. chrysogenum* DS17690

with eight copies of *pen* genes [30], without significant shifting and removing the global regulation of SM and mutation in LaeA.

In order to confirm this hypothesis we carried out fermentation with PAs for WT, HY and E6 strains of *A. terreus* [44]. The LOV production in *A. terreus* is under the control of two major positive regulators, the LovE pathway-specific regulator and LaeA global regulator of fungi SM [44]. The *A. terreus* E6 strain derived from WT by the genetic engineering introduction the additional copy of *lovE* gene under the control of constitutive promotor [27]. LovE is Zn_2Cys_6 transcription factor for pathway-specific regulation of *lov* genes; in *A. terreus OE::lovE* the LOV production increased 10- to 12-fold [27]. Surprisingly, the addition of PAs during the fermentation of the E6 strain led, on the contrary, to a decrease in LOV production (**Figure 5**). But it is also known that pathway-specific regulators can negatively regulate LaeA [49]. For instance, AflR, a sterigmatocystin pathway-specific transcription factor, negatively regulate the expression of *laeA* [49]. E6 strain has the only one targeted change in the genome of WT, that led to constitutive (which also means LaeA-independent) overexpression of *lovE*. The effects of *laeA* downregulation (due to an increase in the dose of the negative regulator gene) on LOV production in E6 strain are compensated by *lovE* upregulation [49]. However, LovE, unlike LaeA, upregulate only *lov* genes for biosynthesis, not for transport and resistance Therefore, the PAs addition during fermentation of E6 strain causes a toxic effect and the LOV production decreases (**Figure 5**).

3.3 The endogenous polyamines content in *A. chrysogenum* HY strain

Since exogenous PAs are able to influence the production of SM in HY strains, it is important to know if there have been any changes in the metabolism and homeostasis of polyamines into the cells of improved fungi strains. Recently it was demonstrated, that *A. chrysogenum* HY strain shows increased resistance to inhibitors

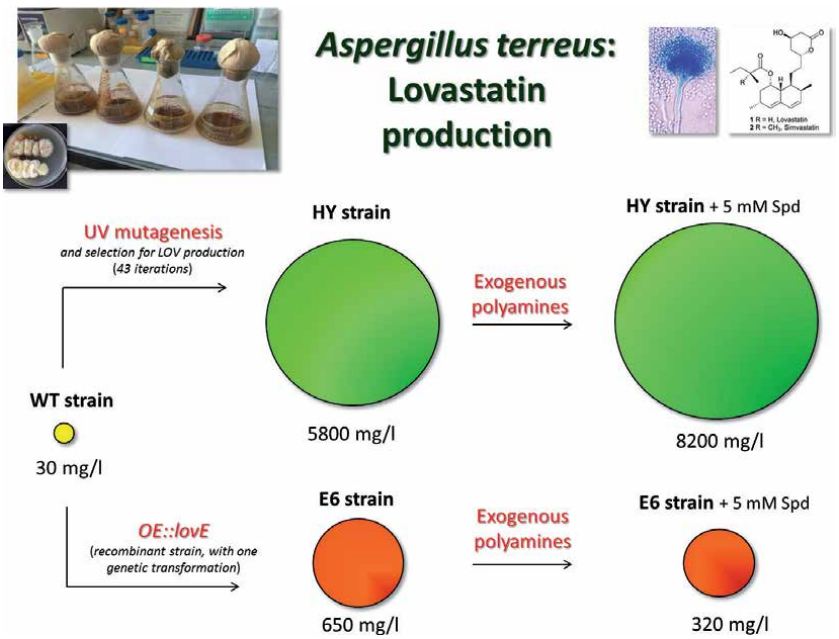

Figure 5.
Effect of exogenous polyamines on lovastatin production in the WT, E6, and HY A. terreus *strains. WT: wild type, E6:* OE::lovE*, HY: high yielding, and Spd: spermidine. Adapted with permission from [44].*

of ODC, the key enzyme of PAs biosynthesis, on minimal agar media [65]. The addition of 5 mM α-difluoromethylornithine (DFMO) or 5 mM of 1-aminooxy-3-aminopropane (APA) completely inhibited the growth of the WT strain, unlike HY strain (**Figure 4**) [65]. Such kind of resistance against inhibiters of key enzyme for PAs production turned out to be rather strange since HY strain is significantly weakened after SCI program [32, 61, 64, 66, 67]. The only previously observed advantage over the WT strain was expressed in CPC overproduction [41]. To explain the phenomenon of the resistance of HY strain to ODC inhibitors, an inhibitory analysis of *A. chrysogenum* WT and HY strains was performed against all pathways of putrescine biosynthesis (**Figure 3**). In filamentous fungi, in addition to the main pathway of putrescine (Put) biosynthesis, via ODC, there is also an additional pathway through arginine decarboxylase (ADC) and biosynthesis of agmatine (**Figure 3**). The inhibitory analysis demonstrated shift from the ADC-dependent to ODC-dependent biosynthesis of Put. During the fermentation for CPC production the total PAs content into HY strain has been increased by about fivefold [65].

The reasons for the increased production of PAs in the HY strain were discussed [65]. One on the reasons may be related to strain improvement techniques. The increasing in PAs content may be spin-o result of mutagenesis and DNA damage. Recently it was demonstrated that PAs can maintain the genome integrity via homology-directed DNA repair, enhancing the DNA strand exchange activity of RAD51 recombinase [87]. PAs also can protect DNA from free-radical damage by reacting direct with the reactive oxygen species [88–90].

4. Conclusions

As a result of CSI programs for filamentous fungi, a number of pharmaceutically significant SMs have been overproduced. One of the side effects of the high yielding strains improvement may be an increase in the content of polyamines (PAs). An increase in the PAs' content could occur as a response to mutagenesis during CSI. The recently discovered increase in the production of targeted SM in some HY strains after the addition of exogenous PAs may occur due to a decrease in endogenous biosynthesis of PAs and the release of additional resources for the biosynthesis of the target SM.

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Conflict of interest

The author declares no conflict of interest.

Notes/thanks/other declarations

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Chapter 3

Generation, Evaluation, and Prospects of Further Use of Mutations Based on New Homozygous Self-Pollinated Sunflower Lines

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Abstract

A majority of sunflower lines and hybrids were based on starting material obtained by traditional methods; so the issues of developing new trends in extending the genetic diversity of this crop require constant attention of scientists. At present, induced mutagenesis along with hybridization has become a leading method for generating new forms of crops. Their success depends largely on availability and assortment of starting material. Induction of mutations is a way to create it. The main value of induced mutagenesis for breeding is determined by opportunities to solve problems that are impossible or difficult to solve by traditional methods. The choice of an effective concentration (dose) of a mutagen is very important, since the frequency and range of mutations depend not only on the mutagen itself but also on its dose and exposure. In addition, it is relevant to search for new mutagens with reduced harmful effects at the same level of mutability. Cytological analysis of chromosomal aberrations is an important method of evaluation and identification of mutagenic effects. In this section, studies into chemical and physical mutagenesis in breeding, exemplified by new modern homozygous self-pollinated sunflower lines, are summarized; methodical recommendations on the use of induced mutagenesis in sunflower breeding are presented; and methods of generation, investigation, and further use of mutations are rationalized.

Keywords: gamma rays, dimethyl sulfate, mutagenesis, meiosis, mutation, self-pollinated line, breeding, sunflower

1. Introduction

In comparison with other oil crops, sunflower produces the highest oil yield per unit area (on average 750 kg/ha in Ukraine), which makes this crop a major oil crop. The oil content in seeds of released hybrids is 50–52%, and in breeding hybrids—up to 60%.

The nutritional value of sunflower oil is determined by high content of polyunsaturated fatty linoleic acid (55–60%), which has a significant biological

activity and accelerates the metabolism of cholesterol esters in the body, which has a positive effect on health. Sunflower oil also contains ingredients that are very valuable to the human body, such as phosphatides, sterols, and vitamins (A, D, E, K). The nutritional value of sunflower seeds per 100 g is as follows: energy, 2445 kJ; proteins, 20.8 g; fats, 51.5 g (of which saturated fats account for 4.5 g; monounsaturated ones, 18.5 g; and polyunsaturated ones, 23.1 g); and carbohydrates, 20 g. This makes sunflower a valuable food product.

Induced mutagenesis allows developing new starting material with various morphological and physiological features and biochemical parameters, increasing the frequency and expanding the assortment of original mutations within a short time.

Mutations are a source of expansion of the genetic diversity of sunflower, which in its turn is a starting material for the breeding of this crop. Radiation and chemical mutagens are used to produce artificial mutations in sunflower [1].

The strongest chemical mutagens (supermutagens), which cause a several hundred-fold increase in the frequency of mutations, include ethyleneamine, diethyl sulfate, dimethyl sulfate, nitrosoethylurea, nitrosomethylurea, hydrogen peroxide, etc. [2].

Since Wetterer's first attempts to gamma-irradiate sunflower seeds in 1911, Shull and Mitchell's experiments in 1933 [3], Soldatov's achievements [4], reports of contemporary scientists Kalaydzhan [5], Lacombe [6], Soroka [7], Cvejic [8, 9], Lyakh [10], Vasin [11, 12], Kyrychenko [13], Škorić [14], and many others, a considerable progress have been achieved in enriching the sunflower gene pool by induced mutagenesis. However, despite considerable advances, due to continuous refreshment of starting material, induced mutagenesis has been and remains an important method for developing new and improving existent starting material in breeding.

Our purpose was to obtain self-pollinated sunflower lines with genetic mutations induced by chemical and physical mutagens that can be used to improve features of the sunflower crop and to develop methodological approaches for studying mutant generations.

2. Means and mechanisms of experimental mutation induction

When researchers obtain and control new hereditary changes in plants during their experiments, some completely new possibilities to create breeding initial material appear.

Since Watson and Crick decoded the structure of DNA, characterized the mechanism of its replication and discovered the system of recording genetic information, highlighting the genetic nature of mutations, it became evident that the primary cause of any mutation is the primary disorders in the DNA structure, which are in the process of cell metabolism can be realized in true mutations or repaired and restored to their original state.

Primary abnormalities induced in hereditary structures of an organism under the influence of natural or artificial factors can cause the appearance of two types of mutations—point ones, caused by disorders in the original structures of the DNA molecule, and chromosomal ones, caused by qualitative or quantitative changes in the chromosomal systems of cells.

Primary disorders in DNA structures are not repaired to their original state; they initiate the processes of gene (point) mutation formation. Such disorders include replacement of nitrogen base pairs (transversions); the inclusion of additional complementary pairs of nucleotides (duplication); loss of nucleotide pairs in the structure of a DNA molecule (deletions); 1800 rotation of nucleotide pairs (inversions), etc.

Initial DNA integrity disorders may result in chromosome rupture. In this case open sections of chromosomal filaments can be combined reaching their original state or form new combinations. Thus, there are chromosomal mutations that are characterized by a wide diversity.

Mutations can attach a molecule of sugar (deoxyribose), phosphate, or a nitrogenous base to the nucleotides. For example, despite the fact that deoxyribose is the only sugar group in DNA, it is not desirable to exclude the possibility of accidental incorporation of individual ribose molecules into DNA. In such cases, the phosphate and nitrogenous bases of the ribonucleotide may be like those of the dezoxyribonucleotides. Phosphoric acid, as a component of DNA and RNA, may contain radioactive P32 atom instead of a normal phosphorus one.

Disorders induced by irradiation in DNA molecules can affect phosphodiester, sugar-phosphate, glycosyl, and other chemical bonds. As a result, single or double-thread breaks occur, as well as the destruction of nitrogenous bases. For example, when dry seeds are irradiated, the nitrogenous bases included in the DNA structure may be converted in to thymine, guanine radicals, etc., which are sufficiently stable in the dry state and sufficiently reactive when wetted.

The alkylating compounds including DMS are a source to introduce the methyl radicals (CH_3), ethyl (C_2H_5), etc. into the molecules, thereby providing an alkylation reaction. They are characterized by a wide range of mutagenic effects, inducing simple and complex substitutions as well as breaks in DNA molecules. All nitrogenous bases, phosphoric acid residue, and even deoxyribose residue are alkylated. As a result of alkylation reactions, the purine bases are most likely to fall out DNA strand, causing the formation of voids at the corresponding points in the molecule. It is obvious that the mechanism of mutagenesis in the alkylation of DNA bases is associated with a violation in the accuracy of the auto-reproduction of DNA molecules.

All these events eventually result in changes within DNA molecules that manifest themselves as mutations, most of which are lethal. However, many mutations are viable. They are involved in the process of gene recombination, and as a result they are integrated in their functions with other genes of the genotype where they originated.

3. Research methodology: selection of starting material

The plant genotype has a significant effect on the specificity and level of mutations. Therefore, selection of starting material plays a significant role in obtaining valuable mutants. Generally, the best area-specific forms are recommended to use as sources, which need refining in terms of individual characteristics and features. Constant self-pollinated forms are the best for mutational breeding, as their mutations can be easily and reliably identified. Therefore, working with mutagenesis, one should apply different methods of isolation of nurseries and mutant plants in order to prevent biological contamination and occurrence, along with mutations, of possible recombinations.

Twelve new homozygous, self-pollinated sunflower lines from a genetic collection of the Plant Production Institute named after V.Ya. Yuriev, which are of breeding value and differ in several morphological and biochemical features, were taken as study objects.

Chemical supermutagen dimethyl sulfate (DMS) and gamma rays were used to induce genetic variability. Two hundred fifty seeds were used in each variant of treatment.

As to chemical mutagenesis, seeds in capron partly loosened sacs were soaked in 0.01 and 0.05% DMS solutions (prepared on distilled water, as some mutagens tend to degrade rapidly in tap water). We prepared these solutions under a hood, wearing rubber gloves: crushed an ampoule with mutagen in water. Depending on the object, the treatment time ranges 2–24 h. With sunflower seeds, the exposure was 18 hours, with periodic stirring. The mutagen/treated seeds ratio (volume/weight) was 10:1. In addition, to accelerate the process of solution penetration through the seed coat, it is recommended to ultrasound seeds for a short time (1–7 min) [15]. After treatment of seeds, in order to reduce the damaging effect, we washed them out for 1 h in running tap water and then sowed in soil on the same day.

Dimethyl sulfate (DMS) is a chemical supermutagen, an alkylating compound, which breaks chromosomes, leading to a large number of chromosomal inversions.

Studying physical mutagenesis on new sunflower lines, we used gamma rays from the radioactive isotope Co60, which has a relatively high irradiation uniformity. Dry seeds were once irradiated on a "Theratron Elit 80" Ionizing Radiation Source Cobalt 60 at Kharkiv Regional Oncology Hospital.

In a research on induced mutagenesis, a great attention is paid to concentrations (doses) of mutagens, which affect the number and quality of mutations; therefore, we used the most effective for agricultural crops DMS concentrations (0.01 and 0.05%) and doses of gamma rays (120 and 150 Gy).

Seeds of corresponding sunflower lines soaked in distilled water were used as controls.

When working with mutagens, which are poisonous and sometimes volatile, one should strictly follow the safety regulations and have appropriate equipment and rooms [16, 17].

4. Generation, evaluation, and further use of mutations: M_1 generation

Mutagen-treated seeds were sown in mutant nurseries: M_1 nursery (area = 20 m^2; single-row plots comprising 250 plants each), M_2 nursery (area = 40 m^2; single-row plots comprising 25 plants each), and M_3 nursery (area = 50 m^2; single-row plots comprising 25 plants each). The sowing scheme was 70 × 25 cm. Seeds were sown with manual planters within the optimal timeframe (2nd–3rd 10 days of May). Winter wheat was the forecrop. Mutant plants in the experimental plots were harvested by cutting and manually threshed.

We observed the expected decrease in the field germinability in the M_1 generation, and the higher concentration or dose of the mutagenic factor was, the more drastically the germinability is reduced. Our data indicate that the phenotypic effect of gamma rays is stronger than the DMS effect.

The highest frequency of phenotypic changes was noticed with 150 Gy gamma irradiation (42.9%); the frequency of phenotypic changes after DMS treatment was only 27–28%. The plant development was delayed and was followed by death. Among the DMS-treated plants, there were no such phenomena; therefore the used concentrations of this chemical are not lethal (**Table 1**).

Mutagenic factors affect biochemical processes in seeds, impairing metabolism and causing unnatural changes, which in its turn influences vital processes in seeds and plants emerging from them. Therefore, studies of microspore formation (meiosis) are a reliable way to investigate the genetic variability of organisms at the cellular level and to the evaluate effects of mutagenic factors on chromosomes of pollen mother cells (PMC) of sunflower lines.

Mutagen	Concentration (%)/dose (Gy)	Number of treated seeds	Number of plants from treated seeds	Number of phenotypically unchanged plants	Number of phenotypically changed plants	Phenotypic effect (%)
Control	No treatment	250	220	220	0	0.0
DMS	0.01	250	218	157	61	28.0
	0.05	250	208	152	56	27.0
Gamma rays	120	250	38	22	16	42.1
	150	250	28	16	12	42.9

Table 1.
Phenotype effect in the M_1 generation of sunflower (average across lines).

Microspores in anthers of flowering plants are the final result of meiosis, which can be traced on temporary and permanent microslides made from immature anthers.

The genotypes of the new self-pollinated lines—sterility fixers (Kh1002B and Kh1008B) and lines— pollen fertility restorers (Kh06134V and Kh201V) pre-treated with chemical mutagen DMS at concentrations of 0.01 and 0.05% or gamma-irradiated at doses of 120 and 150 Gy were studied.

The steps of microslide preparation to investigate chromosomes in sunflower meiosis were as follows:

1. Collection of specimens in the field—calathidium segments (d = 2–3 cm) with anthers. Green star phase.

2. Fixation of the specimens in Clark's solution (absolute alcohol/glacial acetic acid 3:1) for 24 h.

3. Washing out the specimens in 70% ethanol until the odor of acetic acid disappears.

4. Storage of the specimens in 70% ethanol.

5. Staining the specimens in 2% aceto-orcein for 12–24 h. Aceto-orcein solution was prepared as follows: dissolve 1 g of dye in 45 ml of glacial acetic acid and 55 ml of distilled water. Dissolution is carried out in a reflux flask in a water bath for 30–60 min. After cooling, the solution of aceto-orcein is filtered and placed in a glass stoppered bottle. As a part of the study, we demonstrated that aceto-orcein was more effective for staining sunflower chromosomes than acetocarmine.

6. The stained specimen is placed on a mount in a drop of 45% acetic acid or in a drop of 0.5% aceto-orcein, covered with a cover slip and heated above an alcohol burner until boiling.

7. The slide is carefully crushed with a match to get a cell monolayer under the glass and examined under a microscope.

Meiosis was examined under a Micromed XS-5520 microscope at magnification of 40× and 100×. Oil immersion (special immersion oil, cedar oil, or glycerol)

was used to study slides at magnification of 100×. To document and illustrate the results, microphotographs were taken with a Nikon D 3200 kit VR camera equipped with a special Asian Microscope Adapter.

Cells with meiosis disorders were counted by metaphase-anaphase method: the percentage of cells with abnormalities was calculated related to the total number of cells under examination.

Analysis of meiosis in archisporial cells showed considerable effects of DMS and gamma rays on chromosomes in the M_1, which manifested themselves as occurrence of significant chromosomal aberrations compared to the control ($P < 0.99$). The effect level depended on the mutagen exposure.

For example, after DMS treatment, the percentage of cells with abnormalities ranged within 7–14% (0.01%) and 12–20% (0.05%), significantly exceeding the control. After gamma irradiation, the percentage of cells with abnormalities ranged within 16–19% (120 Gy) and 20–25% (150 Gy), significantly exceeding the control.

Comparison of the results showed that the effect of gamma rays on meiosis of the lines under investigation significantly differed ($P < 0.99$) from that of DMS. Gamma rays resulted in the occurrence of more abnormal tetrads in the M_1 compared to DMS treatment. After irradiation, the percentage of abnormal tetrads ranged from 16.00% in line Kh1008B (120 Gy) to 27.10% in line Kh201V (150 Gy), whereas in DMS - treated lines, the percentage of abnormal tetrads ranged from 1.55% in line Kh201V (0.01%) to 21.65% in line Kh1008B (0.05%).

We observed normalization of meiosis and elimination of cells with abnormalities in subsequent mutant generations of sunflower compared to the M_1.

In line Kh06134V, the percentage of cells with abnormalities in different phases of meiosis in the M_2 varied within 8.09–8.69% (0.01 and 0.05% DMS) and within 5.96–8.16% (120 and 150 Gy gamma irradiation). In the M_3, the percentage of aberrations varied within 3.36–4.09% after 0.01 and 0.05% DMS treatment and within 4.29–5.34% after 120 and 150 Gy gamma irradiation.

In line Kh201V, the percentage of cells with abnormalities in the M_2 varied within 4.53–8.45% after DMS treatment and within 7.79–9.48% after gamma irradiation. In the M_3, these values were 2.54–4.96 and 2.15–3.48%, respectively.

In line Kh1002B, the percentage of cells with abnormalities in the M_2 varied within 6.06–4.89% after DMS treatment and within 6.91–7.44% after gamma irradiation. In the M_3, these values were 3.35–4.66 and 3.60–4.83%, respectively.

In line Kh1008B, we noted 4.92–6.95% of cells with abnormalities in the M_2 after DMS treatment and 6.42–10.77% after gamma irradiation. In the M_3, these values were 2.15–3.57 and 3.09–5.26%, respectively (**Figure 1**).

The identified meiotic abnormalities in mutants were manifested as a chromosome lag during the formation of metaphase plate, impaired chromosome distribution in metaphase II, distorted metaphase plates, a chromosome lag in anaphase, asynchronous division during the second stage of meiosis, formation of pentads, triads, dyads, etc. (**Figure 2**).

Note. 1, outsider chromosomes in anaphase I; 2, asynchronous division during the second stage of meiosis; 3, chromosomes outside the metaphase plate in metaphase I; 4, abnormal tetrads.

All the specimens had phenotypic alterations (bent stem, dwarfism, absence of generative organs, chlorophyll deficit, deformation of generative organs, etc.) during subsequent development (**Figure 3**).

To prevent cross-pollination between different sunflower lines, individual inflorescences had been isolated the day before semiflorets opened, the offspring of which were to be examined the next year as the M_2 families. Concurrently, controls, non-treated with mutagens lines, were isolated.

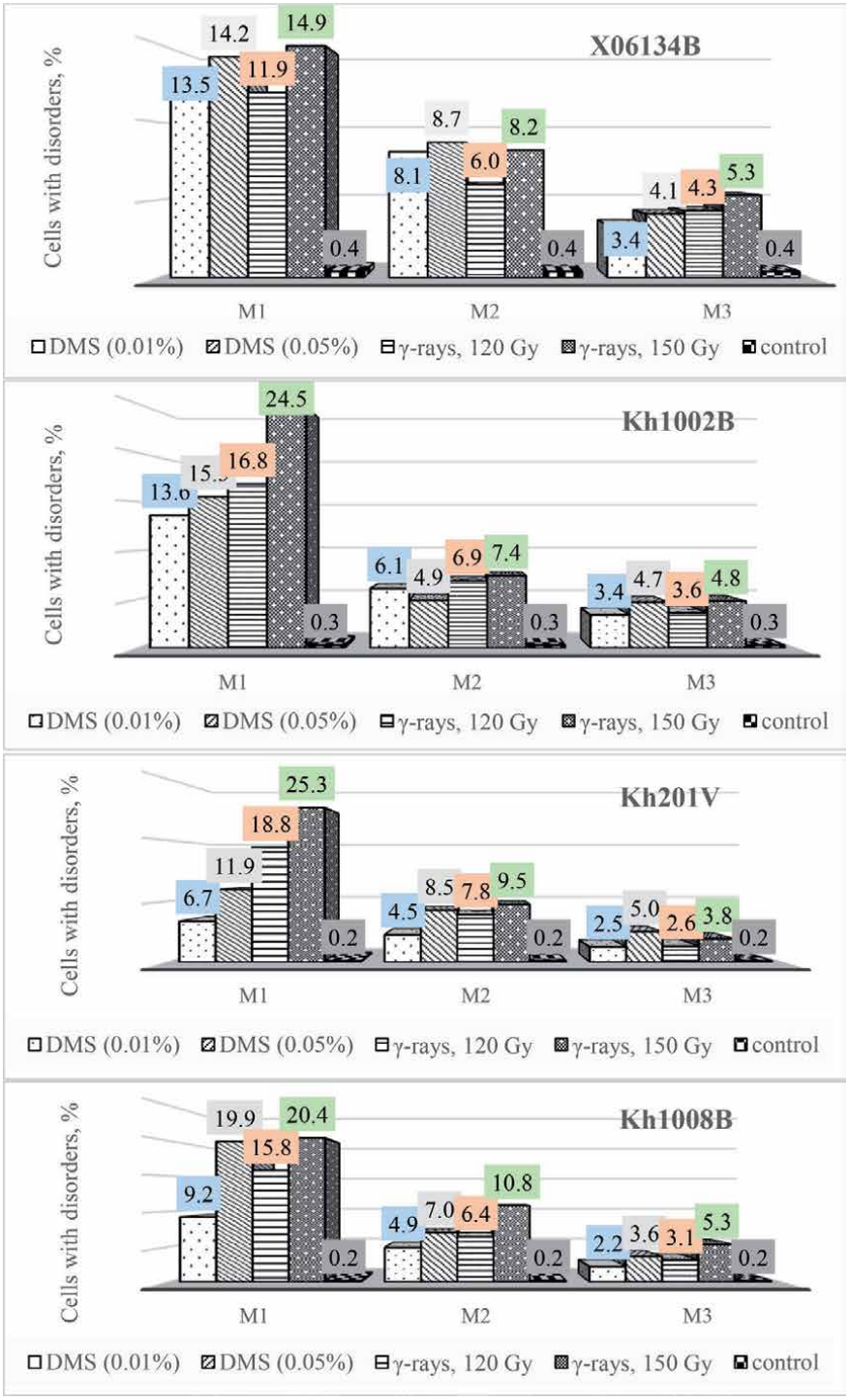

Figure 1.
Normalization of meiosis and gradual attenuation of the mutagenic effects of DMS and gamma rays in the M_1–M_3 sunflower (%).

During the vegetation period, phenological observations of the growth and development of mutant plants were conducted; the field germinability was determined; cytological analysis was performed; and biometric measurements were

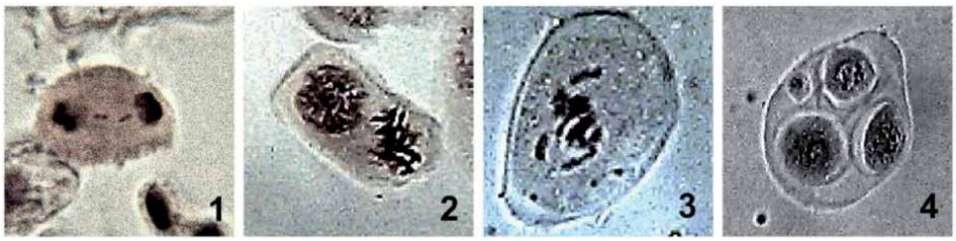

Figure 2.
Microphotographs of meiotic abnormalities in mutant generations of self-pollinated sunflower lines.

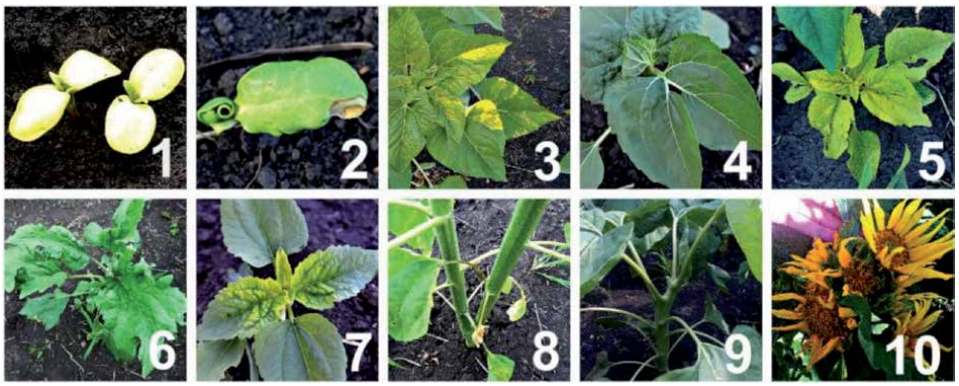

Figure 3.
Phenotypic effects of chemical and physical mutagens in the M_1 sunflower. 1, chlorophyll-deficient shoots from gamma-irradiated seeds (150 Gy); 2, 4, 6, morphoses induced by DMS (0.05%) in the early stages of plant development; 3, 5, 7, chlorophyll morphoses induced by DMS (0.05%); 10, deformation of generative organs induced by DMS (0.01%); 8, stem fasciation induced by DMS (0.05%); 9, absence of generative organs induced by gamma irradiation (120 Gy).

Figure 4.
Morphological changes observed in the M_1 of self-pollinated line Kh06134V: 1, xantha chlorophyll mutation 'golden tip' (0.05% DMS), and 2, "purple tint of leaves" mutation (0.01% DMS).

made (plant height measured 20 days after anthesis, calathidium diameter, and number of leaves per plant). Mutant plants were evaluated for the following parameters: oil content (%), 1000-seed weight, and fatty acid composition of oil.

In the M_1, there were a lot of plants with different phenotypic developmental defects compared to the controls. However, one should keep in mind that most of them were so-called morphoses and consequences of phenotypic variability; such changes are not inherited and disappear in M_2.

It is impossible to detect recessive mutations in M_1 plants, since of 2 alleles of a gene, as a rule, one allele only mutates, and the altered recessive allele is always

paired with the unchanged dominant allele (AA–Aa); therefore, selection of mutations is started with M_2.

Only dominant mutations found by some researchers in some crops (usually in wheat) after exposure to chemical supermutagens can be detected in M_1. Thus, examining the M_1 of self-pollinated line Kh06134V, we distinguished some morphological changes: a chlorophyll-deficient mutation (xantha) called "golden top" (0.01% DMS) and a mutation of a purple tint of leaves (0.05% DMS), which is stably expressed in subsequent mutant generations (**Figure 4**).

5. M_2 and M_3 generations, investigation, and use of mutations

M_2 is sown by families and single plants or by continuous sowing according to variants of mutagenic treatment, with optimal convenient density.

Plants were selected in the second mutant generation by visible morphological and physiological alterations to obtain macromutants as well as well-developed plants without visible alterations to find biochemical mutations and micromutations of quantitative traits. In addition, seeds from families without changes in the M_2 were sampled to reveal them in the M_3.

Analysis of the mutant frequency in the M_2 showed that gamma rays (120 and 150 Gy) produced more plants with alterations than DMS (0.01 and 0.05%). The percentage of plants with alterations after gamma irradiation ranged 36.0–36.4%, while the percentage of plants with alterations after DMS treatment was within 9.6–9.8%. We noted the individual genotypic responses of the lines to the increase in the concentration and dose of mutagens. The rise in the number of plants with alterations in the M_2 depended on the increase in the concentration of DMS and the dose of gamma rays (**Table 2**).

In particular, in line Kh1002B, the total frequency of alterations was 3.2% with 0.01% DMS and 3.5% with 0.05% DMS, whereas plants with alterations were much more numerous with gamma rays (120 Gy–22.6%, 150 Gy–27.8%) (**Table 2**).

0.01% DMS-treated line Kh06134V gave the total frequency of plants with alterations of 14.7%, and the total frequency of plants with alterations after 0.05% DMS treatment was 10.0%. Gamma irradiation produced significantly more plants with alterations: 120 Gy produced 36.6% of plants with alterations, and 150 Gy–47.5% (**Table 2**).

In line Kh1334V, the total frequency of plants with alterations was 3.4 and 3.3% with 0.01 and 0.05% DMS, respectively (**Table 2**).

0.01 and 0.05% DMS produced 8.9 and 13.1% of plants with alterations, respectively, in line Kh201V. However, the effect of gamma rays was more conspicuous, and the total frequency of plants with alterations was 32.1 and 37.5% after 120 and 150 Gy exposure, respectively (**Table 2**).

Most of the alterations observed in the M_2 of the gamma-irradiated lines were nonheritable modifications found in early stages of the plant development, which disappeared during growth, whereas most of the DMS-induced alterations detected in different stages of the plant development were stable. The mutation nature of the changes in the M_2 was finally established by inheritance in the M_3 families.

We studied inheritance of mutant traits in the M_3 and subsequent generations. We also assessed the new mutant lines for breeding value and tested them for economically valuable traits, intending to involve constant valuable forms in hybridization and heterosis breeding in order to obtain new sunflower hybrids.

Having evaluated the alterations, we identified mutants noticeable for oil content in seeds, fatty acid composition of oil, 1000-seed weight, and resistance to the pathogen of sunflower downy mildew.

Original line	Mutagen concentration/ dose	The total frequency of mutations (%)	Frequency of chlorophyll mutations (%)	Frequency of morphological mutations (%)	Frequency of economically valuable mutations (%)
Kh1002B	0.01% DMS	3.2	0.6	1.5	1.13
	0.05% DMS	3.5	0.9	2.1	0.6
	120 Gy γ-rays	22.6	10.7*	7.1*	4.8
	150 Gy γ-rays	27.8*	2.8	13.9	11.1*
	LSD 05	1.5	0.8	1.0	0.8
Kh06134V	0.01% DMS	10.0	1.9	3.3	4.7
	0.05% DMS	14.7*	3.5	5.9	5.3
	120 Gy γ-rays	36.6	3.3	20.0	13.3*
	150 Gy γ-rays	47.5*	16.4*	21.3	9.8
	LSD 05	4.6	2.6	3.3	3.0
Kh1334V	0.01% DMS	3.4	0.6	1.5	1.3
	0.05% DMS	3.3	1.7*	0.7	0.9
	LSD 05	1.4	0.8	0.8	0.8
Kh201V	0.01% DMS	8.9	2.0	4.1	2.9
	0.05% DMS	13.1*	4.4*	5.0	3.7
	120 Gy γ-rays	32.1	5.2	18.7	8.2
	150 Gy γ-rays	37.5*	8.6*	18.4	10.5*
	LSD 05	4.1	2.2	3.1	1.6
Average across 12 lines					
DMS	0.01%	9.6	3.1	3.7	2.8
	0.05%	9.8	3.2	3.8	2.8
γ-rays	120 Gy	36.0	11.3	16.3	8.3
	150 Gy	36.4	8.2	18.9	9.3

** Difference significant at LSD 05.*

Table 2.
Relative frequencies of major mutations induced by DMS and gamma rays in the M_2 sunflower, % (exemplified by 4 lines).

The traits of 1000-seed weight and oil content in seeds followed different patterns, depending on the genotypes of the self-pollinated lines. Thus, in the M_3 there were genotypes, in which DMS treatment and gamma irradiation increased the content of oil in seeds (Od973B, Kh1002B, Mkh845B, X0816B, Kh06135V, Kh1334V, and Kh201V) and 1000-seed weight (Kh808B, Kh1002B, Mkh845B, Kh0816V, Kh785V, Kh1334V, and Kh201V). On the whole, 1000-seed weight insignificantly varied in the M_3 (2–10%).

The mutants with increased content of oil in seeds are listed below: Kh1002B No 224 (0.05% DMS), 50%, and No 876 (150 Gy gamma rays), 48% (46% in the control); Kh0816V No. 422 (0.01% DMS), 50% (53% in the control); Kh1334V No. 609 (0.01% DMS), 48%, and No. 658 (0.05% DMS), 46% (43% in the control); and Kh201V No 685 (0.01% DMS), 54%, and No 1143 (150 Gy gamma rays), 52% (48% in the control) (**Figure 5**).

The mutants with increased 1000-seed weight are listed below: Mkh845B No. 385 (0.05% DMS), 64 g; No. 996 (150 Gy gamma rays), 67 g (48 g in the control);

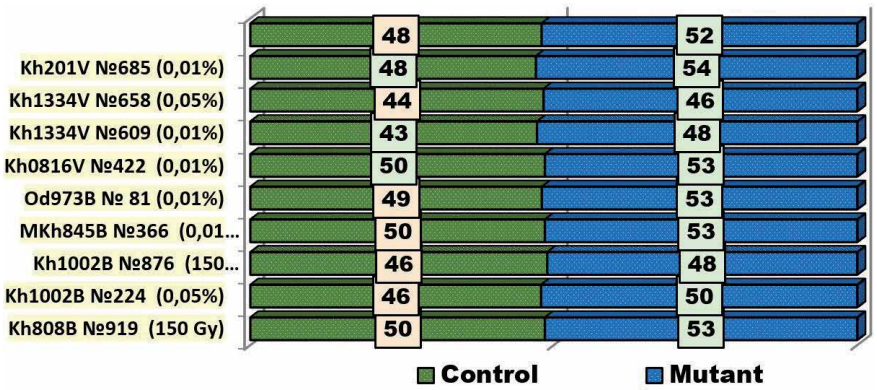

Figure 5.
Oil content in seeds of the mutant families (%).

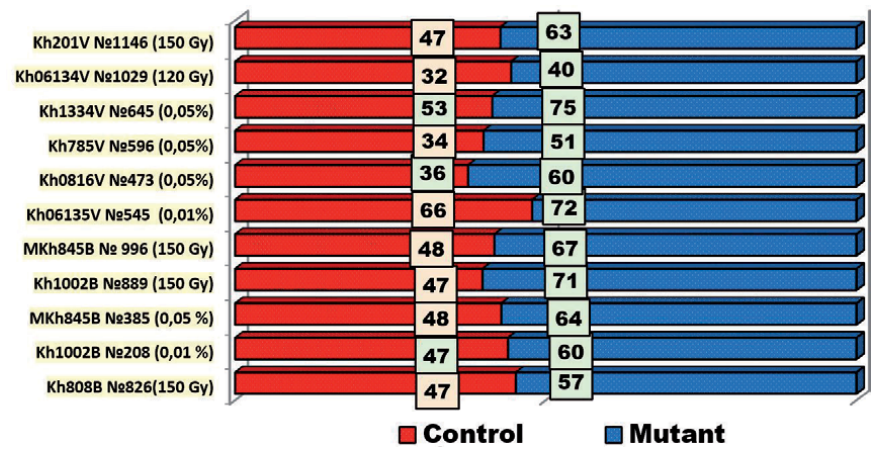

Figure 6.
1000-seed weight in the mutant families (g).

Kh06134V No. 1029 (120 Gy gamma rays), 40 g (32 g in the control); Kh785V No. 596 (0.05% DMS), 51 g (34 g in the control); Kh1334V No. 645 (0.05% DMS), 75 g (53 g in the control); and Kh201V No 1146 (150 Gy gamma rays), 63 g (47.1 g in the control) (**Figure 6**).

Biochemical analysis of oil from mutant sunflower seeds highlighted plants with increased content of linoleic acid of up to 70% (63% in the control) from line Kh201V. Among the mutants obtained from line Kh1334V, there were DMS-induced variants with increased contents of oleic and behenic acids (0.85% vs. 0.64% in the control), and such a combination is valuable for breeding (**Table 3**).

6. Conclusions

As exemplified by the M_1–M_3 mutant generations of sunflower, an important scientific challenge of determining peculiarities of the variability of quantitative and qualitative traits under the influence of DMS (0.01 and 0.05%) and gamma rays (120 and 150 Gy) was theoretically described, and a new solution to it was suggested. The frequency and range of mutational variability in the M_2 were summarized, and the inheritance of the mutant traits in subsequent generations was established. Chromosomal abnormalities in meiosis were characterized, and the

Original line	Mutant	Mutagen, (concentration/dose)	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic	Behenic
			C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C22:0
Kh201V		Control	6.67	0.47	3.87	25.34	62.75	0.28	0.24
	№742	DMS, 0.05%	6.40	0.41	4.95	16.72	70.79	0.15	0.37
	№694	DMS, 0.01%	6.71	0.56	4.00	25.34	62.85	0.13	0.21
	№1133	γ-rays, 120 Gy	7.25	0.80	3.47	17.55	70.54	0.12	0.11
Kh1334V		Control	3.43	0.11	3.78	87.28	3.51	0.35	0.64
	№659	DMS, 0.05%	3.29	0.11	3.54	89.10	2.00	0.30	0.85
	№642	DMS, 0.05%	3.71	0.12	3.52	88.48	2.15	0.30	0.85
	№628	DMS, 0.01%	3.83	0.17	3.85	87.25	2.75	0.47	0.84
	№609	DMS, 0.01%	3.54	0.15	3.40	86.90	3.92	0.35	0.83

Table 3.
Fatty acid composition of oil from the M_3 sunflower seeds.

breeding-genetic values of induced mutations as well as possibilities of their use in breeding were evaluated. Methodological peculiarities of the mutational breeding of sunflower as a cross-pollinated crop were defined, and new mutants with changed features were detected. In addition, this study allowed us to conclude that DMS was more effective than gamma rays for the induction of valuable for breeding mutations in new homozygous self-pollinated sunflower lines.

Thus, induced mutagenesis is a major component of the complex breeding process of creation of new parental lines and hybrids of sunflower with economically valuable characteristics.

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Chapter 4

Nefarious, but in a Different Way: Comparing the Ecotoxicity, Gene Toxicity and Mutagenicity of Lead (Pb) and Cadmium (Cd) in the Context of Small Mammal Ecotoxicology

Peter Vladislavov Ostoich, Michaela Beltcheva and Roumiana Metcheva

Abstract

Lead and cadmium are long established toxic and carcinogenic metals. Still, the mechanisms of their interaction with eukaryotic DNA are not unequivocally understood. New data provide evidence on the influence of both metals on DNA repair, particularly non-homologous end joining (NHEJ) and mismatch repair (MMR). This may help explain the weak direct mutagenicity of both Pb^{2+} and Cd^{2+} ions in the Ames test, as opposed to the proven carcinogenicity of both metals; it has long been proposed that lead and cadmium may induce an imbalance in mammalian systems of DNA damage repair and promote genomic instability. While new evidence for mechanistic interactions of metals with DNA repair emerges, some of the old questions involving dose distribution, pathways of exposure and bioaccumulation/detoxification kinetics still remain valid. To help place the current state of the art in the genetic toxicology of lead and cadmium within the context of ecotoxicology, the current authors propose an integrative approach and offer a review of other authors' work as well as some of their own data on systemic and organ-specific toxicities in laboratory mice. The current chapter is a comparative analysis of the state of the art in the specific toxicity and genotoxicity of Pb and Cd, presenting some new and little-known information.

Keywords: lead (Pb), cadmium (Cd), genotoxicity, ecotoxicology, physiological reactions, DNA damage and repair, cell signaling, laboratory mice

1. Introduction

The last several decades have seen an increase in scientific and public interest in the problem of environmental contamination as a consequence of human activities. A wide variety of chemicals is released into the environment from different sources, either intentionally or as a result of accidents, prompting

widespread concern about the effects of anthropogenic contamination on the biota. While many organic pollutants such as pesticides and petroleum refining products are subject to environmental degradation by physical, chemical, or biological pathways, heavy metals and their compounds typically retain their toxicity over long periods of time. Recently, important advances have been made in the understanding of the gene toxicity and mutagenicity of heavy metals in the environment [1–6]. For instance, it has been established that the gene toxicity of lead (Pb^{2+}) and cadmium (Cd^{2+}) ions is not due to direct DNA-metal interactions [2, 3]. It has been demonstrated that Cd^{2+} affects DNA repair pathways, particularly the non-homologous end joining (NHEJ) of DNA double-strand breaks (DSBs) at very low concentrations (<30 μmol) in several *in vitro* test systems [4, 5]. Nevertheless, some questions regarding the gene toxicity and mutagenicity of lead and cadmium remain open. For instance, several authors have noted that *in vivo* test systems are much more sensitive than *in vitro* systems (i.e., cell cultures) with respect to lead-induced endpoints for genotoxicity assessment (chromosomal aberrations, micronuclei, sister chromatid exchanges, comet assay endpoints) [7, 8]. In practice this means that animal models, especially rodents, are much more suitable for analysis of the genotoxicity of Pb^{2+} than cell cultures. When considering cadmium, useful mechanistic data on mutagenicity and co-mutagenicity has been obtained with *in vitro* test systems [4, 5]. Still, the question of the importance of Cd^{2+} as genotoxic agent in living mammalian organisms remains open. One study has reported lead-induced genomic instability in the progeny of mice exposed to Pb^{2+} *in utero* [9]. It is still unclear if this phenomenon has been observed by other authors and how common heavy metal-induced genomic instability is. If parental exposure to toxic metals can influence the stability of the genome in subsequent generations, this is potentially very alarming and could influence the current standards and permissible limits for occupational and environmental exposure. Last but not least, toxic metals seldom occur alone in contaminated sites. For instance, non-ferrous metal smelters typically emit a cocktail of toxic chemical elements in the atmosphere. This means that an accurate environmental risk assessment should be performed on a case-by-case basis, and that both ecotoxicological biomonitoring, and more general attempts to resolve the problem of heavy metal genotoxicity and mutagenicity, should be concerned not with a single toxicant but rather a plurality of different toxic agents present in a given locality. A number of studies have been performed with wild rodents exposed environmentally to complex contamination including Pb^{2+} and Cd^{2+} [10–23]. While these studies include endpoints for scoring genetic damage (chromosomal aberrations, micronuclei, comet tail length and tail moment) relatively little is understood about the molecular mechanisms underpinning the genotoxicity of complex mixtures of toxic metals.

In summary, from the perspective of ecotoxicology, it is well-established that Cd^{2+} and Pb^{2+} are genotoxic metal ions, especially in complex organisms. At the same time, knowledge about the mechanisms for heavy metal genotoxicity is scarce, with anecdotal evidence for interactions with DNA repair systems in complex vertebrate organisms, and relatively little knowledge of how the gene toxicity of Pb^{2+} and Cd^{2+} fits into the bigger picture of the specific physiological reactions of terrestrial vertebrates to toxic metals. For the purposes of the current study, the main questions regarding lead and cadmium gene toxicity are the following:

1. What are the specific molecular mechanisms, responsible for the gene toxicity of Pb^{2+} and Cd^{2+}? How does intoxication with heavy metals lead to detectable chromosomal damage and mutagenesis? What are the similarities and differences when considering the gene toxicity of lead and cadmium?

2. Can we draw conclusions about the comprehensive biological effects of heavy metals? For instance, it has long been established that terrestrial vertebrates respond to Pb^{2+} and Cd^{2+} by increased expression of detoxifying proteins (metallothioneins) and increased biosynthesis of glutathione. While there is evidence for adaptive responses, how does this apply to genetic damage induced by heavy metals?

3. What are the effects of complex environmental pollution? How do complex mixtures of metallic toxicants affect organisms?

4. Last but not least, what are the prospects, challenges, and potential answers from future studies dealing with the gene toxicity of Pb^{2+} and Cd^{2+}?

In order to provide, in part, answers to these four questions, the current study aims to analyze the state-of-the-art in what is known about the genotoxicity of lead and cadmium within the context of ecotoxicology. The current authors have employed a wide scope of sources in order to synthesize what is currently known and understood about the gene toxicity of Pb^{2+} and Cd^{2+}, and conduct a comparative analysis of the two metals. In addition, insight and information is provided from a personal set of sources and experience, which are not widely publicized. Finally, the current article discusses several potential directions for future studies in the gene toxicity of heavy metals and proposes an integrated, trans-disciplinary approach to solving the problems, associated with the ecotoxicity and gene toxicity of Pb^{2+} and Cd^{2+}.

2. Lead (Pb)

2.1 Ecotoxicity, bioaccumulation patterns, and specific organ toxicities

Lead (Pb) is present in the Earth's crust at comparatively low concentrations (0.121 ppb) and has four stable isotopes (^{204}Pb, ^{206}Pb, ^{107}Pb, and ^{208}Pb) [24]. Although a comparatively rare metal, it has been historically one of the first industrially mined chemical elements. Contemporary sources estimate annual primary production of lead to be 4.6 million metric tons [25]. While Pb has been released in the atmosphere during manufacturing processes and combustion of fossil fuels, leading to global trace contamination, the main concern has been strong local contamination in the vicinity of mining, refining and smelting processes, as well as localized accidental releases. The toxicity of lead has been suspected since ancient times, with authors arguing mass poisoning from the metal in Ancient Rome due to its use for water pipes, glassmaking, and in winemaking processes [26]. Contemporary ecotoxicological research is concerned mainly with local contamination with Pb, with several important impact sites identified in Europe: Bukowno in Poland, Nitra, Slovakia, Asenovgrad, Bulgaria, and the Coto Doñana area in Spain [12, 15, 16, 18, 19, 22, 27–29]. The studies in these areas have dealt mainly with biomonitor species of wild rodents, and have investigated bioaccumulation of lead and other toxic metals, as well as endpoints for the determination of gene toxicity. Regardless of the zoomonitor used (typically, the wood mouse, *Apodemus sylvaticus*, yellow-necked mouse, *Apodemus flavicollis*, bank vole, *Myodes glareolus*, common vole, *Microtus arvalis*, Algerian mouse, *Mus spretus*), similar tendencies for bioaccumulation of Pb in the organisms of small mammals have been detected, and often correlated with the induction of genetic damage (chromosome aberrations, micronuclei). These studies have demonstrated significant effects of heavy metal

contamination on the biota, and have proven the importance of continuing monitoring studies in contaminated ecosystems.

The biokinetics and specific organ and tissue toxicities of Pb have been actively investigated in animal models since the late 1950s, initially employing radioactive tracer isotopes such as ^{203}Pb and ^{210}Pb [30, 31]. This has led to the development of several biokinetic models for the metal in mammalian organisms [31–33]. The Harley-Kneip six-compartent model, developed with the use of primates, is considered to be one of the first informative biokinetic models for lead absorption, distribution and elimination (**Figure 1**).

As evident from the model, a significant percentage of ingested lead (~80%) is excreted without being absorbed by the gut. At the same time, the coefficient for absorption from the bloodstream into bone λ_{12} = 0.34–0.11 is significantly higher than the coefficient for release of Pb from the bones into the bloodstream (λ_{21} = 1.73 x 10^{-3}). In practice, this means that once a significant amount of lead is absorbed into the bones, it is practically impossible to eliminate it. The Harley-Kneip model also emphasizes the differences between juvenile and adult organisms, with juvenile animals much more susceptible to lead bioaccumulation [32]. To a varying level, Pb is also absorbed in the liver, kidneys, and the nervous system. It has been established that, in mammalian organisms, if the metal reaches sustained blood levels above 80 µg/dL, practically every organ and system is affected [24].

The primary targets for lead intoxication are the hematopoietic system, the nervous system and the liver. At sustained blood levels above 50 µg/dL, Pb inhibits

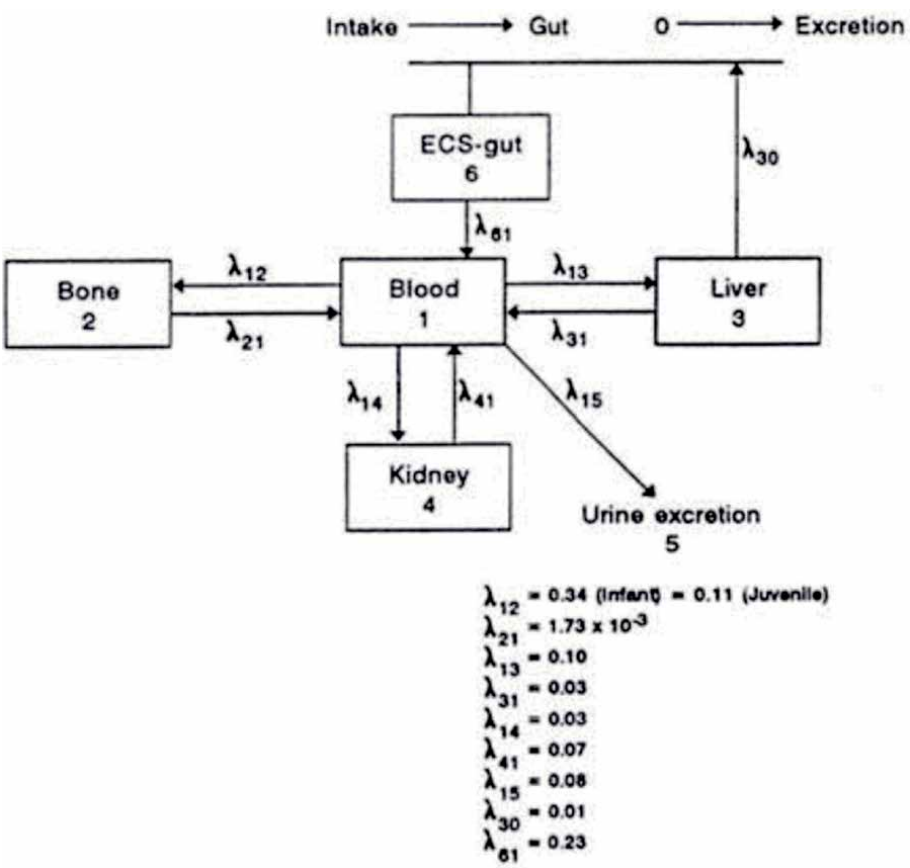

Figure 1.
Biokinetic model for the metabolism of lead in mammalian organisms [32].

the enzymes delta-aminolevulinic acid dehydratase (ALAD) and ferrochelatase, leading to impaired erythrocyte biogenesis, disturbances in erythrocyte kinetics, and anemia [34]. Several authors report an inhibition of the immune system at blood levels above 50 μg/dL, as well as histopathological lesions in the bone marrow at levels exceeding 100 μg/dL [23, 35, 36]. Death from anemia occurs at blood levels above 150 μg/dL. The nervous system is particularly sensitive in young individuals, and it has been established that Pb levels in blood exceeding 18 μg/dL lead to cognitive disturbances; it has been reported that very low doses cause neuronal apoptosis in rats [37]. In cases of chronic and sub-chronic lead intoxication, there is significant liver damage. Macroscopically, the liver increases in size; steatosis, hyperplasia and disruption of the liver microvasculature, as well as focal necrosis, have been observed at doses above 40 μg/dL, with marked changes in the activity of alanine and aspartate transaminase (ALT, and AST) and kidney damage [8].

2.2 Gene toxicity and mutagenicity

Due to low direct mutagenicity levels in the Ames test, lead (Pb) was initially thought not to be directly mutagenic [38]. Nevertheless, evidence soon accumulated that the metal was responsible for producing chromosomal aberrations in occupationally exposed workers and environmentally exposed human populations [31, 35]. Since the 1970s different *in vitro* and *in vivo* studies have been conducted regarding the potential of lead compounds to damage genomic DNA in mammals. The table below presents several informative studies conducted on the gene toxicity of lead using different *in vitro* test systems and endpoints, arranged chronologically (**Table 1**).

The studies cited provide evidence that lead is mutagenic and clastogenic under certain circumstances. While older studies show relatively weak clastogenicity of Pb when considering chromosomal aberrations [39, 40], newer publications report genotoxicity by using more sensitive endpoints, such as the induction of sister chromatid exchanges (SCE), tail length in the comet assay, and induction of γH2AX foci, indicating DNA double-strand breaks [6, 43, 44]. It should be noted that the study indicating the highest toxicity of Pb, uses lead chromate ($PbCrO_4$), which means its effects could be due to the inherent gene toxicity of hexavalent chromium [44].

Several authors have noted the greater sensitivity of *in vivo* test systems when studying the gene toxicity of lead [7, 9]. For the purposes of the current study, several sources dealing with *in vivo* models have been selected (**Table 2**).

It should be noted that, in contrast to *in vitro* test systems not almost all tests with Pb administration to living animals show evidence for genotoxic effects. Not only that, some authors have noted a very close dose dependence of effects on Pb concentrations in living organisms, as well as trans-generational accumulation of chromosomal aberrations after exposure of mice *in utero* [9]. From the viewpoint of ecotoxicology, this means that the risks from environmental exposure to lead compounds are often underestimated when using *in vitro* test systems and only *in vivo* models can provide an accurate assessment of genetic risk to the biota.

Much discussion has taken place concerning the molecular mechanisms of Pb-induced genetic damage. For instance, in the last two decades it has been accepted that lead interferes with the mechanisms for DNA repair, which is evident with studies analyzing Pb as a co-mutagen with other agents such as UV light, X-rays and methylnitronitrosoguanidine (MNNG) [50]. While it is accepter that the metal can inhibit DNA repair, the mechanisms of DNA damage induction *per se* are not well understood. For instance, it has been conclusively demonstrated that Pb and Cd do not interact with DNA directly under physiological conditions [3]. On the other hand, other authors have noted that Pb and other toxic metals can

Authors	Substance tested	Test system	Dose	Exposure	Endpoint	Effect
Bauchinger and Schmid [39]	Lead acetate	CHO cells	10^{-6} to 10^{-3} M	16 h	CA	No effect, except for increase of gaps
Gasiorek and Bauchinger [40]	Lead acetate	lymphocytes	10^{-3} to 10^{-5} M	3 h	CA	No effect
Hartwig et al. [41]	Lead acetate	CH V79 cells	0.5–10 μM	44 h	HPRT mutation	Co-mutagenicity with UV light
					SCE	Increase in SCE
Cai and Arenaz [42]	Lead nitrate	CHO AA8 cells	10^{-6} to 10^{-8} M	48–60 h	CA	No effect
					SCE	Increase in SCE
Wozniak and Blasiak [43]	Lead acetate	lymphocytes	1–100 μM	1 h	Comet assay	Increase in tail length and % tail DNA
Xie et al. [44]	Lead chromate	lung fibroblasts	0.1–5 μM	24–48 h	CA	Increase of % metaphases with damage
					Comet assay	Increased % tail DNA
					γH2AX foci	Dose-dependent increase of γH2AX foci
Pottier et al. [6]	Lead nitrate	EJ30 carcinoma	30–1000 μM	24 h	γH2AX foci	Dose-dependent increase of γH2AX foci
					Telomere score	Telomere instability

Table 1.
Exemplary studies on the genotoxicity of lead compounds in vitro.

induce a pro-oxidative state in living organisms at comparatively low concentrations (<30–50 μmol) [50, 51]. In summary, it can be said that the genotoxicity of lead works at the following levels:

1. Induction of reactive oxygen species (ROS) by Fenton-like reactions; inhibition of key enzymes like glutathione-S-transferase (GST); disruption of lysosomal membranes and induction of apoptosis [51].

2. Induction of genomic DNA damage; inhibition of key DNA repair systems such as base excision repair (BER) and disruption of telomere maintenance [6].

3. Mutagenesis, clastogenesis, tumor initiation and promotion, increase in the levels of apoptosis in some tissues, reproductive toxicity, organ and system toxicities [37].

While the basics of lead genotoxicity have been confirmed, and the metal has been confirmed as reproductively toxic and carcinogenic in mammalian species,

Authors	Substance tested	Test system	Dose	Exposure	Endpoint	Effect
Muro and Goyer [45]	Lead acetate	a/SW mice	1% Pb in food	2 weeks	CA	Increase in CA
Deknudt et al. [46]	Lead acetate	*Macaca fascicularis*	1–15 mg/kg Pb in food	3–16 months	CA	Increase in CA
Sharma et al. [47]	Lead acetate	ICR mice	50–200 mg/kg PB intraperitoneally	Injection	SCE	Increase in SCE
Robbiano et al. [48]	Lead acetate	Sprague-Dawley rats	117 mg/kg in food	3 days	MN test	Increase in MN frequency
Valverde et al. [49]	Lead acetate	CD-1 mice	0.01–1 μM	Inhalation, 3 days	Comet assay	Increase in tail length
Yuan and Tang [9]	Lead acetate	Kunming mice	1 mg/l in drinking water	90 days	Comet assay	Increase in tail length
Tapisso et al. [21]	Lead acetate	*Mus spretus*	21.5 mg/kg Pb in food	17 days	MN test	Increase in MN frequency
					SCE	Increase in SCE

Table 2.
Exemplary studies on the genotoxicity of lead compounds in vivo.

much remains to be investigated regarding the molecular mechanisms of the interactions of Pb^{2+} with mammalian DNA repair systems.

3. Cadmium (Cd)

3.1 Ecotoxicity, bioaccumulation patterns, and specific organ toxicities

Cadmium (Cd) is a malleable, silvery-white metal present in the Earth's crust in concentrations of 01–0.5 ppm, having five stable isotopes (^{108}Cd, ^{110}Cd, ^{111}Cd, ^{112}Cd, and ^{114}Cd) [24]. Discovered as a separate element within zinc ores in 1817, it is a toxicant, associated primarily with the late industrial age. Mined at a large scale since the 1920s, the metal is currently produced at a level of 23,000–24,000 metric tons per year [25]. Similarly to lead, the main concern regarding Cd-associated contamination is local pollution of terrestrial and riverine ecosystems. The toxicity of cadmium was discovered after the start of its extraction from polymetallic ores, with one example being the "itai-itai" disease in the Toyama prefecture of Japan, attributed after 1950 to Cd poisoning [52]. In Europe sites, severely polluted with cadmium are comparatively rare. One exception is the area of Bukowno in Poland, where there is significant local contamination [16, 53, 54]. Several studies deal with the ecotoxicity of Cd with the use of zoomonitors (mainly yellow-necked mice, *Ap. flavicollis* and bank voles, *M. glareolus,* but also the common magpie, *Pica pica*) [53, 54]. While in Europe the element is mostly present as a trace contaminant in cases of polymetallic pollution, the main concern for cadmium contamination are the countries where most of it is mined and produced, namely China, South Korea, Japan, Mexico, Canada and Kazakhstan.

The toxicity of cadmium was discovered after animal studies in the period 1955–1970 [52, 55, 56]. In mammalian organisms, the metal affects primarily the kidneys, liver, pancreas, and, at higher levels, the nervous system [55]. As an established IARC Group 1 carcinogen, Cd increases the risk of lung cancer at low doses, and causes pneumonitis and lung edema at higher doses [52]. Nevertheless, the main target organ for chronic Cd intoxication are the kidneys, where the metal is accumulated, causing proteinuria, hypophosphatemia, histopathological changes in the kidney tissue, and loss of kidney function [57]. High chronic and sub-chronic dose burdens cause histopathological changes in the liver [58, 59]. Due to its antagonistic and antimetabolic activity against necessary elements such as Zn, Cu, and Ca, as well as its interference with a variety of DNA-binding enzymes, cadmium is considered toxic at high levels to all organs and systems [24, 57]. Unlike Pb, which has a strong tendency for bioaccumulation in the animal organism, Cd has higher rates of clearance from mammalian organisms due to the action of metallothionein (MT) proteins—low molecular-weight, highly conserved molecules, which bind non-specifically to dietary elements such as Zn, Se, Cu, as well as toxic elements like Cd, Hg, Ag, As, and, to a much lesser extent, Pb [54, 60]. Metallothioneins bind Cd^{2+} ions in mammals, form Cd-MT complexes, which are excreted through the kidneys, thereby detoxifying, to some extent, low levels of cadmium. Nevertheless, although this system is inducible and upregulated by the presence of toxic metals in the body, it gets saturated at high doses, being unable to compensate high dose burdens of toxic metals [54]. Due to the inefficiency of existing biological detoxication systems, as well as the tendency of the metal for bioaccumulation in plants and animals, Cd is considered very dangerous even at low doses where no physical symptoms are present. It is, therefore, not surprising that a variety of biomonitoring studies for Cd have been conducted [22, 61].

3.2 Gene toxicity and mutagenicity

The debate regarding the genotoxicity of cadmium continued for decades until recently [52]. This was due primarily to the fact that initially, using the Ames test, Cd was demonstrated to have very low mutagenicity. This, on the other hand, contradicted data demonstrating that the metal was a powerful carcinogen in mammals [24, 62]. At the same time, cadmium-induced inhibition of DNA repair systems and, consequently, co-genotoxicity, has been reported consistently since the late 1980s [56, 63]. Due to these relatively early observations on DNA repair inhibition, most *in vitro* studies have focused on the role of Cd as a co-genotoxin when combined with other genotoxic agents, for instance, ionizing and UV radiation, DNA intercalators and DNA alkylating agents [5, 63]. Data on cadmium-induced genotoxicity from several investigations with *in vitro* test models are presented in **Table 3**.

All the studies cited typically provide evidence for co-mutagenicity of Cd with known mutagens such as UV light, DNA alkylating agents such as methylnitronitrosoguanidine (MNNG), and ionizing radiation. Comparably to *in vitro* studies with Pb, older experimental work with cadmium provides evidence for co-mutagenicity (although not direct mutagenicity) of the metal, while newer work, utilizing more sensitive endpoints, provides evidence for specific mechanisms such as DNA repair inhibition [4, 5].

While *in vitro* studies highlight Cd as a powerful co-mutagen due to DNA repair inhibition, several *in vivo* studies have shown that cadmium can be genotoxic (particularly clastogenic) at low doses. The results of several such investigations are presented in **Table 4**.

The *in vivo* studies above demonstrate cadmium genotoxicity at acute sublethal doses. It should be noted that in these studies, no separate co-mutagen is required,

unlike in the *in vitro* models. Even though they prove conclusively that cadmium is genotoxic to mammals, they have a major shortcoming from an ecotoxicological point of view. Namely, the dose administration is either by injection or by oral gavage, which means that the observed effects of cadmium are due to acute exposure, as opposed to chronic and sub-chronic intoxication, which can be achieved by dosing the animal with food, water, or by inhalation means. One of the studies deals with minisatellite DNA instability, demonstrating that Cd intoxication can lead to instability in the non-coding segments of mammalian genomic DNA [69]. Nevertheless, this methodology is still very controversial.

To some extent, the molecular mechanisms of DNA damage induction by Cd^{2+} ions are better understood than those of Pb^{2+}-induced gene toxicity. It has been demonstrated that, at doses above 30 μM, cadmium down-regulates a key system for DNA DSB repair, namely non-homologous end-joining [4, 5]. Evidence suggests that the kinetics and formation of γH2AX foci are impaired at doses greater than 30 μM, with DNA-PKcs catalytic activity falling off at cadmium concentrations at doses of 200 μM [4, 5]. It has been established, as well, that at these doses the metal initially over-activates the system of homologous recombination repair, which may promote genomic instability [4]. Nevertheless, the induction of DNA damage in *in vivo* models by cadmium alone does not show a clear dose-response curve [52]. El-Ghor et al. have demonstrated a significant increase in microsatellite instability in rats exposed to cadmium [69]. Nevertheless, this methodology is controversial, both due to the unknown relationship of microsatellite DNA stability to the overall

Authors	Substance tested	Test system	Dose	Exposure	Endpoint	Effect
Takahashi et al. [64]	Cadmium chloride	*E. coli* CHS26	10^{-8} to 10^{-4} M	4 h	Mutagenicity	β-Gal gene inactivation
Nocentini [56]	Cadmium chloride	Human fibroblasts	10^{-7} to 10^{-2} M	24 h	DNA repair	Inhibition of DNA DSB repair
					DNA synthesis	Inhibition of DNA synthesis
Snyder et al. [65]	Cadmium chloride	HeLa cells	10^{-8} to 10^{-3} M	24 h	UV damage	Co-mutagenicity with UV light
					X-ray damage	Inhibition of DNA DSB repair
Viau et al. [4]	Cadmium chloride, cadmium acetate	HMEC-1 endothelial cells	1–100 μM	24 h	NHEJ activity	Inhibition of DNA DSB repair by NHEJ
					HR activity	Upregulation of homologous recombination
Pereira et al. [5]	Cadmium chloride	ZF-4 zebrafish cells	1–100 μM	24 h	γH2AX foci	Disruption of γH2AX foci kinetics
					Micronuclei	Dose-dependent increase of micronuclei
					DNA repair	Inhibition at doses above 30 μM

Table 3.
Exemplary studies on the genotoxicity of cadmium compounds in vitro.

Authors	Substance tested	Test system	Dose	Exposure	Endpoint	Effect
Mukherjee et al. [66]	Cadmium chloride	Swiss albino mice	0.4–6.75 mg/kg body weight	Injection	SCE	Increase in SCE
					CA	Increase in CA
					MN test	Increase in MN frequency
Privezentsev et al. [67]	Cadmium chloride	ICR mice	1 mg/kg body weight	Injection	MN test	Increase in MN frequency
					CA	Increase in CA
Fahmy and Aly [68]	Cadmium chloride	Swiss albino mice	1–7.6 mg/kg body weight	Injection	SCE	Increase in SCE
					CA	Increase in CA
El-Ghor et al. [69]	Cadmium chloride	Wistar rats	2.93 mg/kg body weight	Oral gavage	Minisatellite DNA	Minisatellite instability
Wada et al. [70]	Cadmium chloride	Sprague-Dawley rats	40–80 mg/kg body weight	Oral gavage	Comet assay	Increase in tail length

Table 4.
Exemplary studies on the genotoxicity of cadmium compounds in vivo.

stability of coding genomic DNA, and the method of Cd intoxication used (oral gavage versus the more common method of administering via food or water). The available literature leads the current authors to believe that cadmium acts as a *tumor promoter*, with initiating events being diverse other factors (ionizing radiation background, metabolic reactive oxygen species, or other genotoxic factors). With respect to reproductive toxicity and cadmium-induced genomic instability, there is reason to believe that cadmium is reproductively toxic at high doses and can cause transmissible genetic damage in the progeny of exposed individuals. Still, much more research (both mechanistic studies and eco-toxicological experimentation) is needed to demonstrate conclusively the potential of the metal to change the genetic structure of exposed populations.

4. Comparing lead and cadmium as genotoxic agents

4.1 Induction of DNA damage

It has been demonstrated that both Pb and Cd do not bind DNA directly, nor induce DNA damage due to DNA-metal interactions [3, 41]. At the same time, it is well-established that the metals promote the generation of reactive oxygen species and interact with redox signaling, disrupting cell homeostasis in organs and tissues

and promoting a pro-oxidative state [41, 71]. In addition, specific target enzymes for Cd^{2+} have been identified—these include specifically several zinc-finger proteins like p53, XPA, PARP-1 and NF-κB. This would indicate increased potential of cadmium ions to act as tumor promoters even at low concentrations [41, 71].

On the other hand, it has been observed that Cd alone, at physiological concentrations, is a more significant causal agent of chromosomal aberrations in *in vivo* models, thereby acting more strongly as a mutagen and clastogen [3]. This is probably due to stronger induction of ROS and disruption of cellular redox signaling [72].

4.2 Interactions with DNA repair systems

Little is understood about the interactions of lead with DNA repair systems. While several studies show disruption of γH2AX foci kinetics and, therefore, disruption of DNA DSB repair, and one study highlights a disruption of telomere maintenance, no mechanistic data exists to suggest how exactly Pb^{2+} ions interfere with DNA repair and the DNA damage response [6, 44].

Much more is known about the influence of Cd^{2+} ions with DNA repair. For instance, the tendency of this metal ion to displace zinc from zinc-finger DNA-binding enzymes leads to a disruption in the nucleotide-excision repair system (NER), which can explain the co-mutagenicity of cadmium with agents such as UV light and DNA alkylating chemicals [56, 63]. There have been a few studies analyzing the effects of cadmium on key DNA DSB repair systems [4, 5]. What these authors have established that, in selected *in vitro* models, even at concentrations lower than 30 μM, cadmium chloride inhibits non-homologous end-joining (NHEJ), over-activates the MRE-11-dependent homologous recombination (HR) and telomere maintenance, and leads to a general disturbance in γH2AX foci kinetics (a very sensitive indicator for DNA damage and repair), as well as a sharp decrease in DNA-PKcs catalytic activity, indicating inability to repair double-strand breaks.

While cadmium has undoubtedly been better studied as a genotoxic and co-genotoxic agent, lead (Pb) is also a significant genotoxin, albeit at significantly higher concentrations (>10-fold or more). Pointing out the exact mechanisms of the interaction of Pb with mammalian DNA repair system remains a valid topical area for future research.

5. Gene toxicity of lead and cadmium in the context of ecotoxicology

Mechanistic studies, both *in vivo* and *in vitro*, are informative when trying to understand the basic principles of heavy metal genotoxicity. Nevertheless, what is the significance of environmental exposure to Pb and Cd? Typically environmental exposure occurs chronically or sub-chronically through food, drinking water and inhalation, and happens at comparatively low doses. In addition, exposure patterns are complex. For instance, pollution is often polymetallic, with an added variety of other organic and inorganic chemicals. Studies have been conducted in localities where pollution from lead-zinc smelters and mines is present, such as Asenovgrad in Bulgaria and Bukowno in Poland [10, 12, 14, 16, 18, 22, 27] as well as in areas, polluted by ecological accidents [15, 19].

The answers that these studies give us is that each studied locality has its own pollution pattern, leading to its own "fingerprint" of systemic toxicity and gene toxicity. For instance, it has been demonstrated that for BALB/c laboratory mice, exposed to 1% polymetallic industrial dust through food, the contents of the heavy

metals Pb and Cd increase steadily in a 90-day experiment, while at the same time the incidence of chromosome aberrations peaks at the 45-day midpoint, indicating the possibility of an adaptive response [18]. Similar results have been obtained with wild rodents from the same locality in different time frames [20]. Another area of research, which is currently active and productive, is heavy metal detoxification, particularly with the use of zeolite sorbents [29]. From the viewpoint of ecotoxicology, it is already known how chronic and sub-chronic doses of Pb and Cd affect the organism separately, but more research (including mechanistic studies) is needed in order to understand the effects of complex pollution patterns on living organisms.

The available data on the gene toxicity and eco-toxicity of Pb and Cd leads the current authors to believe that more significant research needs to be done in two main areas:

1. Mechanistic studies dealing with the specific effects of the two metals on DNA repair systems. This is especially true for Pb, since lead-induced chromosomal aberrations in mammalian cells at low doses are a well-established fact, but no concrete mechanistic studies on the effects of Pb on DNA repair systems have been conducted.

2. Ecotoxicological studies highlighting the effects of different cocktails of pollutants in a given locality on a standardized test system. Suitable *in vitro* systems, which have been proposed include metabolically competent human and rat hepatoma cell lines, which have been used for the study of metabolically activated genotoxins for over two decades [73].

Finally, connections should be made to existing occupational safety and environmental legislation regarding the use of Pb and Cd worldwide. Some of the safety concerns regarding the two elements stem from the fact that heavy metals and their compounds are highly persistent in the environment. Additionally, gene toxicity, especially in the case of cadmium, have caused EU authorities to propose banning the use, mining and refining of Cd within the EU entirely. Since effects of Pb and Cd on genomic instability in the progeny of mammalian species have been observed [9, 69], but are not well understood, it is advisable that safety approaches to Cd and Pb have a "conservative approach," meaning that exposure tolerance limits and environmental releases should be as low as possible in order to mitigate risk to humans and the biosphere.

6. Conclusion

The current work has analyzed the state-of-the art in what is known about the gene toxicity of lead and cadmium in an ecotoxicological context. Cd has been demonstrated as a powerful co-mutagen in *in vitro* test systems and as a direct mutagen *in vivo*. While Pb is generally a less potent inductor of chromosome aberrations, it has still been demonstrated to be genotoxic, particularly *in vivo*. While many studies have been conducted on the environmental exposure to Pb and Cd and their compounds, the interactions of the two metals as genotoxic agents are not yet fully understood. Two main challenges remain for future research in ecotoxicology and toxicogenetics: the combination of mechanistic *in vivo* and *in vitro* studies with ecotoxicological research, in order to understand better the specific pathways of heavy metal-induced gene toxicity, and future research on the detoxication of Pb and Cd and the mitigation of their gene toxicity.

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Chapter 5

DCLK1 and DNA Damage Response

Janani Panneerselvam, Dongfeng Qu, Courtney Houchen, Michael Bronze and Parthasarathy Chandrakesan

Abstract

Genome integrity is constantly monitored by sophisticated cellular networks, collectively termed as the DNA damage response (DDR). The DDR is a signaling network that includes cell cycle checkpoints and DNA repair and damage tolerance pathways. Failure of the DDR or associated events causes various diseases, including cancer. DDR is primarily mediated by phosphatidylinositol-3-kinase-like protein kinase (PIKKs) family members ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR). However, one of the many unanswered questions regarding these signal-transduction pathways is: how does the cell turn the DDR signals on? There was no conclusive demonstration of the involvement of a specific sensory kinase in DDR signals until our recent research on the DCLK1 role in regulating ATM after genotoxic injury. Currently, various studies are demonstrating the importance of DCLK1 in DNA damage response. Here, we discuss the novel insights into the role of DCLK1 in DNA damage response.

Keywords: DNA damage, DDR, ATM, ATR, DCLK1

1. Introduction

DNA damage exists in all cellular organisms, and DNA, the genetic material in each living cell is the fundamental unit of life and its integrity and stability are essential to life [1]. However, DNA is not passive; rather, it is a chemical unit subject to be attacked from a range of endogenous and environmental damaging agents. The endogenous damages are the damage caused by reactive oxygen species or metabolic byproducts, and DNA metabolization; exogenous damages are caused by external agents, like radiations, toxins, chemicals, and microorganisms [2]. In response to the DNA damage, cells rapidly recruit a sophisticated network which is called DNA damage-response (DDR) systems. DDR systems include DNA repair mechanisms, damage tolerance processes, and cell-cycle checkpoint pathways [3]. Failure of DDR causes genomic instability which results in various diseases including immune deficiency, neurological degeneration, premature aging, and severe cancer susceptibility [2, 4]. Indeed, great progress has been made towards understanding the mechanisms of DDR in homeostasis, carcinogenesis and cancer advancement but much remains to delineate how the DDR network systems are regulated. Furthermore, how the DDR network is formed and how it is fine-tuned by upstream and downstream mediators or signaling pathways that support the homeostasis or disease progression required to understand. While the rapid activation of DDR against the

DNA damage is expected, it is unclear how and who activates or gives the instruction to DDR network systems? Gaining knowledge about DDR and its regulators will not only enhance our understanding of DDR functions but will undoubtedly giving us opportunities to better manage human diseases. Although, very few studies reported that protein kinases and DNA adaptor molecules or DNA regulators may influence or send signals to DDR after DNA gets damaged [5, 6]. DCLK1 is a member of the protein kinase superfamily and the doublecortin family, that belongs to the group of microtubule-associated proteins [7]. Our novel findings that DCLK1 regulate DNA damage response and cell survival following genotoxic injury opens many windows of how DDR is regulated [8]. In this chapter we will highlight the functional role of DCLK1 in injury, DDR and cell survival, which will lead us to a better understanding of DCLK1 expression in helping genomic stability in normal and neoplastic cells.

2. DNA damage, DNA damage response, and DNA repair

DNA is the source of genetic information in all living cells, its integrity and fidelity are essential to life. Because DNA is not passive, it is a chemical entity subject to be assaulted from various reactive agents, causing DNA damage [9]. DNA damage can be subdivided into two types: (1) endogenous damage caused by reactive oxygen species (ROS) that are derived from metabolic byproducts and (2) exogenous damage caused by radiation (UV, X-ray, gamma), hydrolysis, plant toxins, and viruses, chemical toxins [9, 10]. Most of the DNA damage can be repaired by the host systems called the DNA damage response (DDR) and DNA repair systems. Such systems also face failure and not 100% efficient, which resulted in either cell death or cell survival with un-repaired DNA causing mutation and eventually cancer [11]. In some cases, the un-repaired DNA damage accumulates in non-replicating cells, such as neurons or myocytes of adult mammals, and can cause aging [12]. The DDR is a sophisticated cellular network, which constantly monitors the integrity of the genome, in response to DNA damage [13]. Once the DDR gets activated it rapidly recruit downstream protein sensors and adaptors establishing the sensing, activating repair, tolerating damage and apoptosis (**Figure 1**). DDR is primarily mediated by phosphatidylinositol-3-kinase-like protein kinase (PIKKs) family members, ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [13, 14]. The ATM pathway for homologous recombination (HR) repair is activated after a double-stranded break. The ATR pathway for nonhomologous end-joining (NHEJ) is associated with single-stranded DNA and stalled DNA replication forks. ATM pathway is a higher-fidelity repair pathway than the ATR. For lesions repaired by the HR, double-strand breaks (DSBs) are detected and processed by the MRE11–RAD50–NBS1 (MRN) complex [15, 16]. For lesions repaired by the NHEJ, DNA breaks are detected and the process by the ATR interacting protein (ATRIP) complex. ATM and ATR transduce the most upstream DDR signal by phosphorylating the checkpoint kinases CHK1/CHK2 and the tumor suppressor protein p53, which resulted in cell cycle arrest to allow time for DNA repair. The main function of DNA-PK activated under the ATM/ATR pathway is to induce cell cycle arrest and DNA repair [17]. DNA repair is a vital cellular process required for the maintenance of genomic integrity and fidelity [18]. Living cells employ several DNA repair pathways for distinct types of DNA damage. There are five major DNA repair pathways: (1) mismatch repair (MMR), (2) nucleotide excision repair (NER), (3) base excision repair (BER), (4) homologous recombinational repair (HR), and (5) non-homologous end joining (NHEJ) [19, 20]. MMR's primary

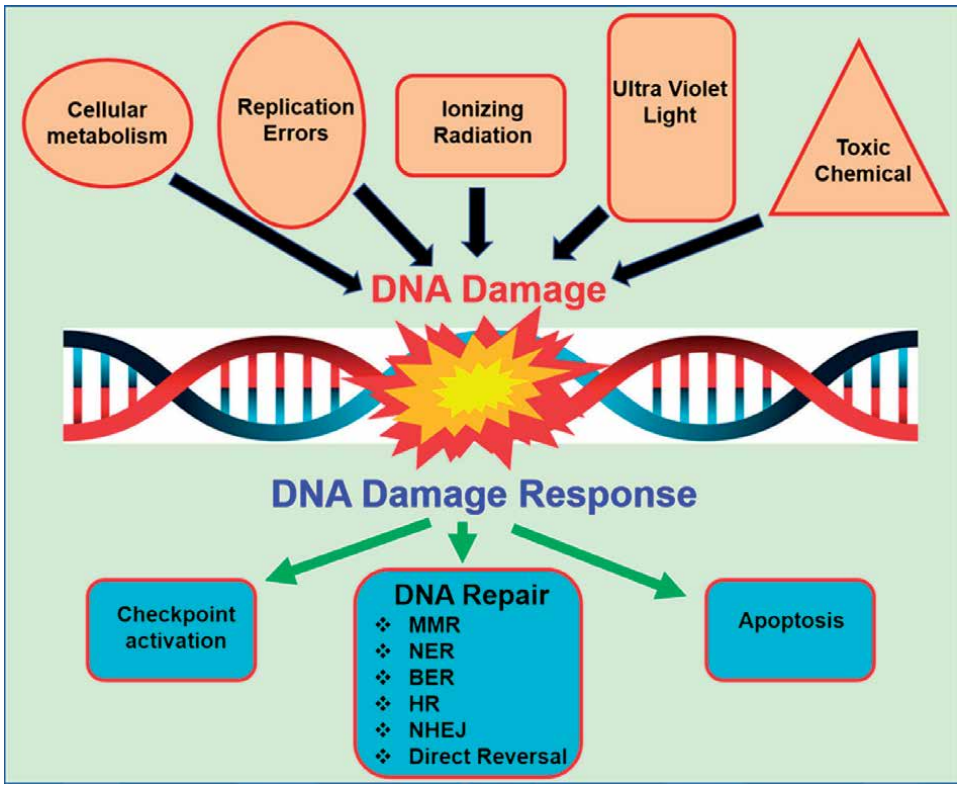

Figure 1.
DNA damage, DNA damage response, and repair. Graphical illustration demonstrating the DNA damage caused by different sources and the cellular response to DNA damage.

function is to remove base mismatches and small insertion and deletion loops which is introduced during replication. The NER pathway is a multistep process that serves to repair a variety of DNA damage, including DNA lesions caused by UV radiation, toxic chemicals, or chemotherapeutic drugs that form huge DNA adducts. BER primarily repairs non-bulky lesions produced by alkylation, oxidation or deamination of bases. The BER pathway deals with base damage, the most common insult to cellular DNA. DSBs can be repaired by either HR or NHEJ. HR uses a homologous DNA template and is highly accurate, whereas NHEJ rejoins the broken ends without using a template and is often accompanied by loss of some nucleotides. Direct reversal of DNA damage is one repair mechanism used to restore damaged DNA without using excision, resynthesis, and ligation [21, 22].

3. DCLK1

The human doublecortin (DCX) gene family comprises members that share the tubulin-binding domain and known to have limited functions in microtubule-associated regulation and neuronal-regulation [23]. One of the best known and most interesting members of this DCX family is doublecortin-like kinase 1 (DCLK1 also known as DCAMKL1), a gene encoding for a protein that is 70% identical to doublecortin in the microtubule-binding N-terminal domain. However, unlike doublecortin, the DCKL1 gene also encodes for a serine–threonine kinase C-terminal domain that is similar to Ca^{2+}/calmodulin-dependent protein kinase II but lacks a canonical calmodulin-binding site [24, 25]. DCLK1 gene also encodes

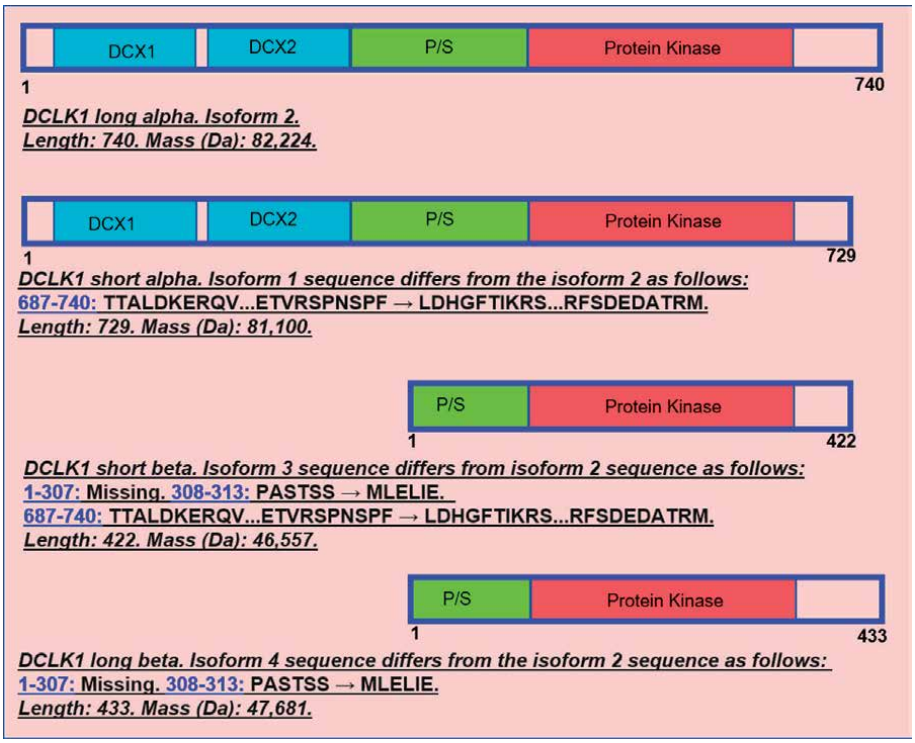

Figure 2.
Human DCLK1-isoforms. Graphical illustration demonstrating the length of each isoform and shared protein kinase domain between DCLK1 isoforms referenced in UniProt; www.uniprot.org/uniprot/O15075. DCX1 = Doublecortin1; DCX2 = Doublecortin2; and P/S = pro/Ser rich domain and a protein kinase domain.

for a serine/proline-rich domain in between the doublecortin and the protein kinase domains, which mediates multiple protein–protein interactions. In humans, DCLK1 consists of four primary isoforms with a shared kinase domain-driven from two promoter regions termed α and β (**Figure 2**) [26–28]. The α-promoter drives the expression of isoforms termed α-long (isoform 2) and α-short (isoform 1) which contain an N-terminal microtubule-binding region with high homology to DCX. Importantly, the α-promoter isoforms are specifically expressed in the DCLK1+ tuft cells that eventually give rise to tumor stem cells following relevant mutagenesis in the colon and pancreatic cancer [29–31]. The β-promoter drives the expression of two isoforms termed β-long (isoform 4) and β-short (isoform 3) that can be used to predict survival in colon cancer [32]. Although these isoforms likely play a significant role in tumorigenesis through their kinase activity, there is no evidence that they are functionally involved in the regulation of DDR, until our first report to demonstrate its direct interaction with ATM.

4. DCLK1 and DDR following injury and inflammation

Cell survival after severe injury requires highly coordinated complex interplay between the diverse molecular signaling responses to repair the injury [15, 33]. We discussed three fundamental standards about the critical role of DCLK1 in intestinal epithelial cell survival after severe genotoxic injury: (1) how intestinal epithelial cells respond to severe DNA damage because intestinal epithelial cells are the most affected cells after bone marrow during radiotherapy or accidental or incidental radiation exposure [34] and (2) how DCLK1 a kinase protein expression

play key role in injury response, because DCLK1 expressing cells survive high dose radiation and DSS-induced inflammation [29]. It is reported that the deletion of DCLK1 (Villin^{Cre};DCLK1$^{f/f}$ mice) in the intestinal epithelial cells does not confer a significant deleterious phenotype in adult mice, compared with their wild-type littermates [35]. However, after 24 h of 12 Gy total body irradiation (TBI), none of the intestinal epithelial-specific DCLK1 knockout mice survived longer than 5 days [35]. The best-known primary defense mechanism against the genotoxic injury-induced DNA damage is the DDR, which repairs the damaged DNA and increased the survival of intestinal epithelial cells [36]. Indeed studies demonstrated that deficient DDR has been suggested to increase intestinal epithelial death and loss of survival [37]. During the early event of DNA damage, the ATM-H2AX axis gets activated, generating gamma-H2AX and other adaptors, providing a stage for efficient homologous recombinant repair [38]. Recently, ATM knockout or loss of Rad50 and Mre11 was reported to increase intestinal injury and lethality [39, 40]. But how these DDR signaling pathways were regulated following radiation injury is not well known. Chandrakesan et al. reported that the absence of DCLK1 expression in the intestinal epithelial cells abrogated the activation and expression of ATM, gamma-H2AX, and downstream adopter proteins BRCA1, Rad50, and MRE11 in the intestinal epithelial cells 24 h post-TBI [8]. Furthermore, it is reported that this reduction persisted up to 3.5 days post-TBI. It is suggested that there is a profound defect in intestinal DDR in DCLK1 knock-out mice, which

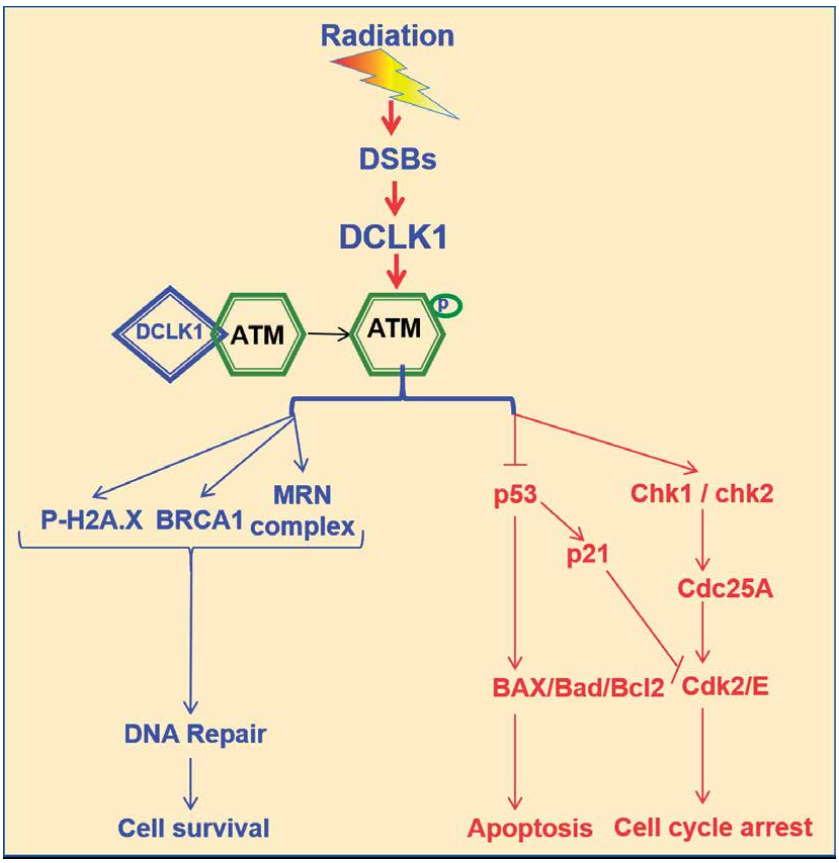

Figure 3.
DCLK1 and DDR. Graphical illustration demonstrating the regulatory role of DCLK1 in DDR following radiation injury.

might contribute to defective epithelial survival and overall survival. Interestingly they established that phosphorylation of ATM which is critical for its activation is reduced in the intestinal epithelial cells of DCLK1 knock-out mice, under physiological conditions, and discovered that DCLK1 can directly interact with ATM for its activation. ATM activation during and or after radiation injury directly depends on the ratio of DCLK1-ATM interaction [8]. Furthermore, DCLK1 knockdown and overexpression experiments with the YAMC cell line in vitro established that DCLK1 interaction is important for ATM activation. It is the first study to establish a direct link between DCLK1 and ATM mediated DDR, for the survival of cell in response to severe genotoxic injury (**Figure 3**).

5. DCLK1 in the regulation of DDR in cancers

A faulty DDR system can initiate cancer development [41]. Cancer cells with a DDR deficiency are profoundly dependent on remaining DDR [42, 43], for example in the case of ATM deficiency cancer cell relies on the ATR pathway. Therefore, DDR inhibition in cancers exploits these defects by inhibiting the remaining DDR system, and which in turn causes cancer cell death. Indeed the healthy cells are not vulnerable to DDR targeted therapies because normal cells can have higher expression of DDR only if they exposed to injury [44, 45]. Most cancer cells depend on their enhanced DDR activation for their survival, mainly activation of ATM and ATR pathways, and associated, CHK2, histone H2AX, and p53 [46, 47].

The present conventional radiotherapy and chemotherapies including platinum-based therapies are used to kill cancer cells by inducing DNA damage. A huge problem that arises when using conventional therapies is the development of resistance by these cancer cells whose DDR repair the genomic instability, which causes conventional therapies to fail [48, 49]. Cancer cells as a short-term solution can bypass the DNA damage caused by chemotherapeutic agents by a mechanism known as translesion synthesis [50, 51]. Cancer cells with high DNA damage tolerance allow DNA replication to proceed in the presence of DNA damage include the convergence of adjacent replicons, re-priming of DNA synthesis downstream of lesions on the leading strand and discontinuous synthesis of Okazaki fragments on the lagging DNA strand [52, 53]. Given the fundamental role of DDR in the gain of chemo-resistance, the novel strategies of combination therapies including DDR targeted therapies will be effective [41]. Recent regulatory approval of olaparib (Lynparza), a poly (ADP ribose) polymerase (PARP) inhibitor, which inhibits PARP enzyme activity and forms severe DSBs [54]. In cancers, PARP inhibitor increases genomic instability that results in tumor cell death [55, 56]. Although, the pharmacological inhibitors of PARP have shown promising results in preclinical studies and in clinical trials, the gain of resistance in cancer cells to PARP inhibitors, is inevitable [57]. However, the combination of PARP inhibitors with other DDR agents including ATR inhibitors, CHK1 inhibitors, ATM inhibitors, and DNA-PKs inhibitors, or with chemotherapeutic agents are novel strategies currently investigated to overcome resistance to PARP inhibitors [57] (**Table 1**—[58]). However, while the DDR targeted therapies are expected to cause DNA damage in tumor cells, it is unclear how these DDR networks are regulated in cancer cells? DDR regulators in cancers are reported recently, (1) MORC2 (MORC Family CW-Type Zinc Finger 2) is required for DNA damage-induced PAR production and PAR-dependent DNA repair signaling cascades and stimulates chromatin remodeling [59, 60]. Inhibition of MORC2 in breast cancer cells impaired DDR and sensitize cancer cells to PARP inhibitors. (2) MYB is an oncogene that plays an important role in regulating DDR in ER+ breast cancers and inhibition of MYB induces DNA

ClinicalTrials.gov identifier	Title	Phase	Drug target
NCT02797964	A Phase 1 Trial of SRA737 in Subjects with Advanced Cancer	I	CHK1 inhibitor
NCT02797977	A Phase 1 Trial of SRA737 in Combination with Gemcitabine Plus Cisplatin or Gemcitabine Alone in Subjects with Advanced Cancer	I	CHK1 inhibitor Chemotherapy
NCT03057145	Combination Study of Prexasertib and Olaparib in Patients with Advanced Solid Tumors	I	CHK1 inhibitor PARP inhibitor
NCT02516813	Phase 1 Trial of MSC2490484A, an Inhibitor of a DNA-dependent Protein Kinase, in Combination with Radiotherapy	I	DNA-PK inhibitor Radiotherapy
NCT03308942	Phase 2, Multi-Arm Study of Niraparib Administered Alone and in Combination with PD-1 Inhibitor in Patients with Non-Small Cell Lung Cancer	II	PARP Inhibitor PD-1 Inhibitor
NCT02660034	The Safety, Pharmacokinetics and Antitumor Activity of BGB-A317 in Combination with BGB-290 in Subjects with Advanced Solid Tumors	I	PARP Inhibitor PD-1 Inhibitor
NCT02264678	Ascending Doses of AZD6738 in Combination with Chemotherapy and/or Novel Anti Cancer Agents	I	ATR Inhibitor Chemotherapy PDL-1 Inhibitor
NCT01844986	Olaparib Maintenance Monotherapy in Patients with BRCA Mutated Ovarian Cancer Following First Line Platinum Based Chemotherapy. (SOLO-1)	III	PARP inhibitor
NCT02282020	Olaparib Treatment in Relapsed Germline Breast Cancer Susceptibility Gene (BRCA) Mutated Ovarian Cancer Patients Who Have Progressed at Least 6 Months After Last Platinum Treatment and Have Received at Least 2 Prior Platinum Treatments (SOLO3)	III	PARP inhibitor
NCT02446704	Study of Olaparib and Temozolomide in Patients With Recurrent Small Cell Lung Cancer Following Failure of Prior Chemotherapy	I	PARP inhibitor
NCT02789332	Assessing the Efficacy of Paclitaxel and Olaparib in Comparison to Paclitaxel/Carboplatin Followed by Epirubicin/Cyclophosphamide as Neoadjuvant Chemotherapy in Patients with HER2-negative Early Breast Cancer and Homologous Recombination Deficiency (GeparOla)	II	PARP inhibitor Chemotherapy
NCT02264678	Ascending Doses of AZD6738 in Combination With Chemotherapy and/or Novel Anti Cancer Agents Drug: administration of AZD6738 in combination with carboplatin Drug: administration of AZD6738 Drug: administration of AZD6738 in combination with olaparib Drug: administration of AZD6738 in combination with MEDI4736	I/II	PARP inhibitor Chemotherapy

ClinicalTrials.gov identifier	Title	Phase	Drug target
NCT00535353	AZD2281 and Irinotecan in Treating Patients with Locally Advanced or Metastatic Colorectal Cancer	I	PARP inhibitor Chemotherapy
NCT00678132	AZD2281 and Cisplatin Plus Gemcitabine to Treat Solid Tumor Cancers	I	PARP inhibitor Chemotherapy
NCT00515866	Study to Assess the Safety & Tolerability of a PARP Inhibitor in Combination with Gemcitabine in Pancreatic Cancer	I	PARP inhibitor Chemotherapy
NCT01460888	Radiotherapy & Olaparib in COmbination for Carcinoma of the Oesophagus (ROCOCO)	I	PARP inhibitor Radiotherapy
NCT02308072	Phase I Study of Olaparib Combined with Cisplatin-based Chemoradiotherapy to Treat Locally Advanced Head and Neck Cancer (ORCA-2)	I	PARP inhibitor Chemoradiotherapy

Selected clinical studies involving DDR inhibitors as a single agent or adjuvant therapy in combination with conventional chemotherapy or radiotherapy in cancer patients.

Table 1.
Ongoing DDR inhibitor trials.

damage and tumor cell death [61]. (3) IKKα directly activates ATM via BRAF regulates DNA damage and inhibition of IKKα induces DNA damage associated cell death in colon cancer [62]. Although these signaling molecules are involved in the regulation of DDR in cancers, their mechanism and therapeutic efficiency are yet to develop.

DCLK1, a protein kinase is overexpressed in various tumor cancers [63–65]. DCLK1 plays a critical role in injury response for repair via regulating DDR [8]. However, recently the role of DCLK1 in the regulation of DDR in cancers has established by many investigators [66–68]. In an in vitro mechanistic study, it is reported that DCLK1 caused chromatin instability, and chromatin rearrangement in colon, lung, and breast cancer cell lines, which drives the advancement of cancer cells for progression and this function is independent of its kinase activity [68]. In another study, it is reported that DCLK1 regulates the phosphorylation of CHK1 in pancreatic cancer cells. Inhibition of DCLK1 enhanced the sensitivity to gemcitabine treatment [67]. In a parallel study, it is shown that DCLK1 by regulating the phosphorylation of CHK1 enhances the sensitivity of 5-FU in colon cancer [69]. Taken together these reports suggest that DCLK1 plays a critical role in the regulation of DDR for cancer cell survival and progression. Novel therapies in the combination of targeting DCLK1 along with chemotherapeutic agents or targeting DCLK1 plus targeting an ATM or ATR with chemotherapeutic agents will be beneficial for the most effective treatment against cancers particularly the resistant cancers.

6. DCLK1 and radiation mitigators

Radiation therapy has been used for the treatment of a wide range of malignancies, especially cancers. Radiation not only kills cancer cells, but it also kills/affects normal healthy cells. Exposure of normal tissues to a substantial amount of radiation may cause both acute and chronic damage that can result in adverse effects for intended treatment [70, 71]. For example, radiation enteritis (RE) is an

intestinal inflammatory process that occurs in response to radiotherapy [72]. It is a major health concern characterized by abdominal pain, diarrhea, and rectal bleeding [73]. It can be complicated by translocation of gut bacteria into the circulation due to the loss of intestinal epithelial cells, disruption of intraepithelial tight junctions, and loss of regenerative ability resulting in severe impairment of gut function and even death. Relatively little is known about the mechanisms underlying the intestinal epithelial injury repair, cell survival and crypt regeneration in RE. Besides the severe side effects resulted in gastrointestinal mucosal damage, ionizing radiation also impairs the bone marrow-derived hematopoietic cells and immune response, which causes a significant increase in morbidity and mortality [74]. Prevention and amelioration of radiation-induced adverse effects would improve the quality of life for patients and would help cancer curability by allowing more intense therapies [75].

There are three types of chemical/biological agents used to interfere with radiation effects. Agents used before or at the time of radiation treatment are called radioprotectors, whereas agents used post-treatment are called radiation mitigators, agents used to ameliorate established normal tissue toxicity are considered treatment [76]. Currently, Amifostine is the only radioprotector in clinical use, and a few radiation mitigators been used [76]. DCLK1 can be a novel target for radiation mitigators for action, as it is mentioned above, deletion of DCLK1 within intestinal epithelial cells results in the premature death of mice following severe radiation injury, suggesting that DCLK1 is a major mediator of the crypt epithelial survival to severe genotoxic injury via a DDR-ATM mediated mechanism [8]. Recently, single-cell analysis in the intestine has revealed that the DCLK1 expressing epithelial cells in the intestine is the primary source of Cox1 (Ptgs1) and Cox2 (Ptgs2) for PGE2 synthesis [77]. PGE2 increases the survival of murine intestinal stem cells when given before photon radiation [78, 79]. It is reported that the treatment of dimethyl-PGE2 to the intestinal epithelial cells increased the survival of the colonic epithelial cells by enhancing DCLK1 expression and reduced the DNA damage [8]. Qu et al. reported that Notch signaling in the intestinal epithelium prevents the death of epithelial cells expressing DCLK1 following radiation injury [80]. Also, dietary pectin has been demonstrated to increase intestinal crypt stem cell survival following radiation injury via a DCLK1 [81]. Kantara et al. have reported that a novel regenerative peptide TP508 can significantly increase survival and delay mortality by mitigating radiation-induced intestinal and colonic toxicity, and its mechanism of action via upregulating the expression of DCLK1 in the intestinal epithelial cells which are responsible for maintaining and regenerating intestinal crypts [82]. In summary, DCLK1 could be a potential radiation mitigator by regulating DDR to ameliorate radiation-induced adverse effects.

7. Conclusion

It is becoming clear that DCLK1 contributes to DNA damage response and repair via direct and indirect mechanisms that are distinct from its role as a stem cell marker. A long-standing question of how DDR is regulated in response to DNA damage is now getting a new clarity. Furthermore, (i) DCLK1 and ATM direct interaction for ATM activation following DSBs and (ii) radiation mitigators enhance the survival of cells following DSBs via a DCLK1 dependent mechanism, which expects that DCLK1 can be a potential target for radiation mitigators in radiotherapy. Finally, in the expanding field of DDR, it is important to consider how DCLK1 is involved in the repair of DNA in cancer and homeostatic injure conditions. This will allow clinical and non-clinical researchers and practitioners to

avoid possible issues with DCLK1 therapeutics, such as enhanced cancer survival and cancer advancement with DCLK1 dependent mitigators during radiotherapy, and, more excitingly, inhibition of DCLK1 along with DDR following chemotherapy or radiotherapy in cancers will lead the way to develop novel strategies for the effective treatment of cancer.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

Oxidative Stress and Vanadium

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Abstract

Air pollution is a worldwide health problem, and metals are one of the various air pollutants to which living creatures are exposed. The pollution by metals such as: lead, cadmium, manganese, and vanadium have a common mechanism of action: the production of oxidative stress in the cell. Oxidative stress favors the production of free radicals, which damage biomolecules such as: DNA, proteins, lipids, and carbohydrates; these free radicals produce changes that are observed in different organs and systems. Vanadium is a transition element delivered into the atmosphere by the combustion of fossil fuels as oxides and adhered to the PM enters into the respiratory system, then crosses the alveolar wall and enters into the systemic circulation. In this chapter, we will review the oxidative stress induced by vanadium—as a common mechanism of metal pollutants—; in addition, we will review the protective effect of the antioxidants (carnosine and ascorbate).

Keywords: air pollution, metals, vanadium, oxidative stress damage, ascorbate, carnosine

1. Introduction

Air pollution is a worldwide concern because of the health problems associated with its uncontrolled emissions that affect all the biological systems. Within the wide range of pollutants, the suspended particles or particulate matter (PM) are of particular interest, which became more important since IARC listed them as carcinogens. The toxicity of PM is the consequence of the elements adhered to its surface [1]. An example of this are the particles generated by the combustion of fossil fuels and its derivatives, these particles usually consist of a carbon core on which complex mixtures of compounds are adhered, such as: polyaromatic hydrocarbons, toxins, sulfates, nitrates, and especially transition metals like vanadium, manganese, chromium, among others [2]. Metals are considered to play an important role in the induction of toxic effects reported in the literature [3].

Metals are the largest category of globally distributed pollutants with a tendency to accumulate in some human tissues and with a high toxic potential at relatively low concentrations. Constant exposure to certain metals has been linked to inflammation, cell damage, and cancer [4]. Each metal has its own mechanisms of action [5]. Some of them produce its adverse effects alone, while others interact with various factors resulting in greater damage in different organs and systems [4]. It is known that metals, including vanadium, have different toxic pathways, and oxidative stress is the most frequent mechanism [5].

Oxidative stress is the consequence of an imbalance between the production of free radicals and the antioxidant capacity of an organism [6]. It may result from the increase in exposure to oxidants, due to the decrease in the protection against oxidants, or because both events occur simultaneously [7].

A free radical represents any chemical species of independent existence that has one or more missing electrons spinning in its external atomic orbitals. This electrochemical configuration is unstable and gives them property of being a highly reactive and short-lived chemical species [8]. Most of the free radicals of biological interest are usually extremely reactive and have a very short life span (microsecond fractions). When a radical reacts with a non-radical compound, it results in other free radicals, thus generating chain reactions that produce biological effects [9], coupled with the fact that when they collide with a biomolecule and subtract an electron (oxidizing it), it loses its specific function in the cell [8].

Regardless of the origin, free radicals can interact with the biomolecules found in the cell such as nucleic acids [10], proteins, lipids, and carbohydrates [9], thereby causing potentially serious modifications and consequences in the cell [10].

Vanadium is an element that is find in various oxidation states and participates in reactions that lead to the production of free radicals such as superoxide, peroxovanadyl, and the highly reactive radical hydroxyl [8].

2. Oxidative stress, vanadium, and cellular and systemic damages

The increasing production of free radicals leads the cell to an imbalance in its redox state and thus generating oxidative stress; therefore, the cellular dysfunction will be reflected in the failure of organs and systems.

2.1 Oxidative stress and cellular damage

The cell is the basic functional unit of life and its dysfunction induced by oxidative stress might produce DNA damage and cell death.

2.2 Oxidative stress, vanadium, and DNA damage

The International Agency for Research on Cancer lists vanadium pentoxide (V_2O_5) as "a possible carcinogen for humans" in group 2B. The above was based on evidence of lung cancer generation in mice that was published by the National Toxicology Program [11]. However, evidence on the carcinogenicity of vanadium has been widely questioned by Duffus in 2007 [12] and Starr et al. [13]. Information related to the carcinogenic and genotoxic potential of vanadium pentoxide (V_2O_5) is limited [14]. In both animal and human models, the effects on the DNA caused by vanadium include single strand breaks, micronuclei, chromosomal aberrations (structural and numerical), and oxidation of nitrogenous bases [15, 16]. The spectrum of alterations that DNA might have as a consequence of free radicals interaction, in this case caused by vanadium, are: deoxyribose oxidation, modification of

nitrogen bases, chain cross-linking, and ruptures [6]. The double or single chain breaks that are generated by the interaction of free radicals with DNA are produced by the fragmentation of the sugar-phosphate skeleton or indirectly by the cleavage of oxidized bases [17].

The above indicates that vanadium is an element with mutagenic potential, because its genotoxic, aneugenic, and clastogenic effects, although there are not strongly data supporting that vanadium is carcinogenic, this possibility should not be eliminated, because the DNA damage caused by the exposure and therefore genetic instability, processes closely related to the generation of malignancy [18].

2.3 Oxidative stress, vanadium, and cellular death

Cell death is central to physiological homeostasis; the balance between cellular differentiation, proliferation, and death support aspects of biology, including embryogenesis, organ function, tissue remodeling, immune regulation, and carcinogenesis. Cell death was once believed to be the result of one of three different processes: apoptosis, autophagy or necrosis; however, in the last decade about 15 different types have been reported, highlighting that a cell can die via different pathways which depends on the intensity of the stimuli [19]. Reactive oxygen species (ROS) activates cell death and plays different roles in the biological systems which can be either injurious or beneficial. Generation of ROS might be caused by metals such as: arsenic, cadmium, chromium, cobalt, copper, gold, iron, nickel, rhodium titanium or vanadium [8]. Vanadium compounds can interconvert into different species (vanadyl and vanadate) event which is constantly occurring inside the cell in the presence of ROS [20].

Studies *in vivo* and *in vitro* showed that vanadium compounds induce cell death in leukemia [21], lung cancer [22] cervical and breast carcinoma [23], neuroblastoma [24], liver carcinoma [25], osteosarcoma [26], and pancreatic ductal adenocarcinoma [27]. *In vitro* studies demonstrated that the cell lines stimulated with vanadium compounds produce H_2O_2 and O_2 and induce autophagy, necroptosis, and mitotic catastrophe [27]. Apoptosis is the main type of cell death associated with vanadium compounds, reporting the release of cytochrome c from mitochondria [21] and the disruption of the mitochondrial membrane potential [25]. This type of cell death is mediated through the activation of p53 and p21 [27], which modulate the activation or inactivation of phosphorylation of some proteins such as MEK, ERK 1/2, PI3K, p38, JNK, TNF-alpha, and NFkB [28].

2.4 Oxidative stress and vanadium in different systems

The systemic vanadium effects observed *in vivo* and *in vitro* are briefly described below.

2.4.1 Reproductive system

The reprotoxic effects of vanadium in male reproductive system in laboratory animals include interruption of spermatogenesis [29], morphological and biochemical changes in spermatogenic cells [30], abnormalities in the shape and movement of sperm, as well as decrease in the weight of the testis, epididymis, and prostate [31].

One of the mechanisms of vanadium toxicity includes imbalance in the cellular redox state [30]; testicular cells are highly susceptible to free radical actions because its membranes are rich in polyunsaturated fatty acids, which limits the fluidity of the membrane and alters the functioning of integral membrane proteins [32].

In rat's testis, after given sodium metavanadate ($NaVO_3$), an increase in malondialdehyde (MDA) was found, which is a product of lipid peroxidation, as well as a decrease in the activity of superoxide dismutase (SOD) and catalase [33]. Intraperitoneal administration of $NaVO_3$ caused in the testis a decrease in the number of germ cells, the presence of degenerated cells, and necrosis of the seminiferous tubules, associated to the increase in testicular lipid peroxidation and inhibition of the activity of antioxidant enzymes (SOD and catalase) [34]; alteration in spermatogenesis, decrease in serum testosterone, LH and FSH levels, inhibition of steroidogenic enzyme activity, increase in testicular vanadium concentration, inhibition of antioxidant enzymes (SOD, catalase and GPx), increased levels of lipid peroxidation [29], and abnormalities in the form of sperm have also been reported [35].

During female reproductive processes, such as ovarian follicle development, ovarian steroid synthesis, ovulation, fertilization, and implantation, require certain amounts of ROS [36]; however, due to the oxidizing effects of vanadium, the delicate balance between ROS generation and the cellular antioxidant system can be altered.

In the case of the female reproductive system of rats, it has been observed that the administration of vanadium sulfate ($VOSO_3$) causes oxidative stress and biochemical alterations in uterine cells, such as the decrease in the activity of alkaline phosphatase and adenosine triphosphatase; while in the ovary, the damage of the oocyte and ovarian follicles was observed, as well as stromal fibrosis [37]. In an inhalation model of vanadium in non-pregnant females, histological alterations were found in the ovary and uterus and lipid peroxidation, indicated by the increase in the levels of 4-hydroxynonenal (4-HNE) a marker of oxidative stress [30].

Vanadium crosses the placental barrier and exerts its toxic effects on embryos and fetuses; in rats, it has been observed that fetal weight decreases and the number of implants and fetuses, while the number of resorptions, malformations, and dead fetus increases [31]. The fetotoxic and embryotoxic effects of vanadium have also been associated with oxidative stress since both in fetuses and in mothers exposed to vanadyl sulfate ($VOSO_4$), and lipid peroxidation was observed in the liver [37].

2.4.2 Urinary system

Kidney chronic disease (CKD) has increased worldwide. The main risk factors for the development of this disease are diabetes, metabolic syndrome, and hypertension. However, there are a segment of population that has none of these risk factors and there are other factors that are being studied as a possible cause of renal injury. One of them is the environmental pollution, particularly pollution by metals in atmosphere and water. Arsenic, cadmium, mercury, lead, and vanadium have been reported as nephrotoxic metals because of the production of ROS and the induction of oxidative stress. These metals enter the body by oral or inhaled exposure, then they are absorbed, enter into the systemic circulation, and distributed into the organs where they may accumulate. Finally, most of them are eliminated by the kidney, reason why this organ is one of the most affected structures by metals [38]. Also, there are reports that in CKD when there is a problem to eliminate pollutant metals, these can concentrate into kidney cells and the damage worsened when it occurs in humans, both in children and adults [39]. Oxidative stress and inflammation are the principal mechanisms of renal injury; in addition, arsenic, cadmium, mercury, and lead are associated to hyperglycemia that may aggravate the oxidative stress and the renal damage. Vanadium is an exception because it has a hypoglycemic effect, but this does not ameliorate its toxicity [40].

Vanadium is nephrotoxic, as it has been proved mainly in animal models, but also in humans [41]. In a report of human acute poisoning by oral ammonium metavanadate, hypoglycemia, bronchoconstriction, and acute renal insufficiency were the causes of death; in a chronic model of vanadium exposure reported glomerulonephritis, glomerular and tubular necrosis that lead to renal insufficiency and hypertension [42]. The reported findings in other study with ammonium metavanadate *p.o.* at doses of 30, 45, and 60 mg/kg were edema, vacuolization, and degeneration of epithelial tubular cells at 21st day of exposure [43]. Another research group, using different compounds and doses of vanadium (45 and 90 mg/kg) reported thickening of glomerular basement membrane, pyknotic nuclei, cellular vacuolization, and pyelonephritis [44]. In our group, in a subchronic model of vanadium inhalation, we found foci of inflammatory cells, vacuolation, loss of microvilli of epithelial tubular cells, and changes in urine parameters as proteinuria and hematuria associated to the increase, in a time dependent manner, of 4-hidroxynonenal (4-HNE) [45] (**Figure 1A** and **B**). Oxidative stress is also the toxic vanadium mechanism reported by other groups, for example, Marouane et al. [46] found lipid peroxidation, protein denaturation, DNA degradation, and cell membrane disintegration; in addition, Scibior et al. [47] reported elevated malonaldehyde (MDA) as a marker of oxidative stress and enhanced total antioxidant status in a rat model of 12-week oral sodium metavanadate exposure.

2.4.3 Immune system

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines whose function is to distinguish between self and non-self-antigens in the host system, thus providing mechanisms against infections and tolerance to the components of the host. When an antigen attacks the host, two distinct, yet interrelated, branches of the immune system are activated, the nonspecific/innate and specific/adaptive immune response. Both of these systems

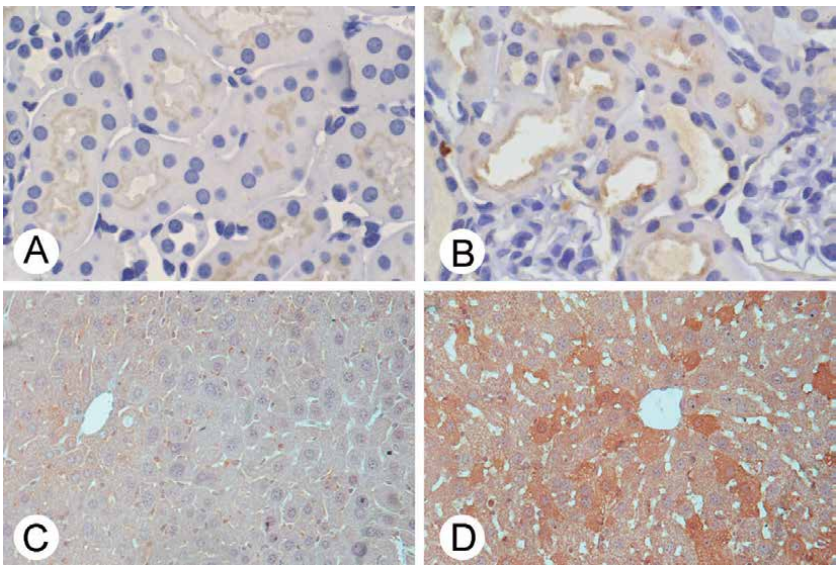

Figure 1.
4-hydrxynonenal (4-HNE) in kidney and liver as a marker of oxidative stress. (A) Kidney tubules in control group with a basal 4-HNE immunoreactivity. (B) In vanadium group, 4-HNE immunoreactivity increased in microvilli of proximal tubules after 8th-week exposure. (C) Liver of control group with a basal 4-HNE immunoreactivity. (D) Liver of vanadium group after 8th-week exposure with increase in 4-HNE immunoreactivity in hepatocytes, some of them with a very intense mark.

have certain physiological mechanisms, which enable the host to recognize foreign materials as foreign materials and to neutralize, eliminate, or metabolize them [48]. The immune system is a target of air pollutants, such as vanadium that might impair its function and induce oxidative stress.

In previous studies, effects from vanadium inhalation on the immune system have been demonstrated. Changes in the spleen morphology and a decrease in humoral immune responses have been reported [49], as well as a decrease in the number of thymic dendritic cells, its expression of CD11c and MHC-II biomarkers, and an increase of thymic medullar epithelial cells [50]. Oxidative stress could be an important mechanism involved in these effects and some mechanisms are described as follows:

Sodium metavanadate ($NaVO_3$) induced oxidative stress through generation of ROS and depletion of the antioxidant defense systems. When the exposure is chronic, the oxidative stress turns out in severe damage [51].

The effect of vanadyl sulfate ($VOSO_4$) in blood glucose and in the spleen, in streptozotocin (STZ)-induced diabetic rats was evaluated. The levels of lipid peroxidation (LPO) and glutathione (GSH) in the spleen were analyzed. After 60 days of treatment, spleen LPO significantly increased, but spleen GSH levels significantly decreased in the diabetic group. On the other hand, treatment with $VOSO_4$ reversed these effects in STZ diabetic animals [52]. These studies show that vanadium induced oxidative stress in the spleen, which might disrupt the immune response.

2.4.4 Digestive system

The liver as the major site for metabolism, biotransformation and detoxification of drugs and foreign compounds, is constantly exposed to ROS resulting in oxidative stress and frequently, permanent and irreversible tissue damage [53]. Studies have shown that liver is one of the most important target tissues for vanadium toxicity with its capacity to form ROS by interacting with mitochondrial redox centers, mainly in mitochondrial respiratory processes I, II, and III [54]. Studies with HepG2 cell line have shown that exposure to vanadium causes damage to nuclear and mitochondrial DNA, as well as decreased cell viability [55]. *In vivo* studies from our group demonstrate that vanadium increases lipid peroxidation in V-exposed animals [56]. **Figure 1C** and **D** show the oxidative marker 4-HNE in liver parenchyma.

As a heavily irrigated, highly connected organ with neural, endocrine, digestive, absorptive, and immune functions, the gut can enter oxidative cycles mostly by two well-defined mechanisms:

1. Ambient-polluting microparticle swallowing: especially in heavily polluted areas (industrial centers, cities, mines, etc.), the air is charged with carbon PM, whose size varies between 10 and 2.5 (or even less) micrometers. Such particles are normally covered by metals (vanadium, for instance), which get trapped via natural defense mechanisms in the nasal and oral mucosa, slowly, descending into the pharynx and into the digestive tract carried on through saliva [30].

2. Direct toxic ingestion: recent research relates ingestion of food ingredients—especially sugar (sucrose or high fructose) present mostly in sugar-sweetened beverages (SSB)—with tissue damage. Although there is no specific data on gut tissue damage, it has been reported in other bodily systems—e.g., kidney [45]. This represents a particularly severe problem in a world where no matter the country, the SSB consumption increases steadily year after year [57].

Research on this matter has still a long path to walk. However, preliminary results from ongoing protocols at our laboratory show a significant rise in 4-HNE levels in the gut epithelium in response to air pollution and SSB consumption mice models, which indicate higher oxidative stress levels vs. control groups.

2.4.5 Cardiovascular system

Air pollution has been associated to thrombosis and cardiovascular risk. Pollutants, including PM and metals may induce oxidative stress and inflammation predisposing to endothelial dysfunction, platelet activation, and procoagulant state [58]. There is epidemiological evidence that elevated concentrations of pollutants, e.g., vanadium, are associated to an increase in ER visits for acute cardiovascular effects or exacerbations of preexisting cardiovascular diseases [59].

Vanadium induces oxidative stress, and there is evidence of their toxic effects on endothelium, platelets, and myocardium. An *in vitro* study using HUVEC (human umbilical vein endothelial cells) exposed to different V_2O_5 concentrations reported an increase in ROS that damaged endothelial cells leading to apoptosis and diminished proliferation. This might be involved in endothelial dysfunction and increased cardiovascular risk associated to metals [60]. An *in vivo* vanadium inhalation mice model, from our group, reported thrombocytosis that is an increase in platelet number, but also the presence of giant platelets that are associated to increase reactivity [61]. Also, we found a megakaryocytosis with an increase in megakaryocytes size and granularity because of the activation of JAK/STAT pathway [40, 62, 63]. Platelet aggregation after subchronic vanadium inhalation diminished, but activation markers of platelets P-selectin or CD-62p were increased after the 4th week of exposure, maybe because of the slow elimination of vanadium, so it is possible that this metal has on platelet aggregation a long-term effects [64]. Another effect of vanadium on cardiovascular system is arrhythmia; in our group, we studied its effect on myocardium N-cadherin and connexin-43, important proteins in the intercalated discs. The reduction of both proteins and its effect on the electric stimuli conduction was proposed to explain the pathophysiology of the arrhythmias induced by vanadium [65]. Vanadium and other metals induce oxidative stress that may damage several cells of cardiovascular system.

2.4.6 Respiratory system

The lung is one of the main targets of air pollution damage because it is the first site in contact with the pollutants suspended in the air. After reaching the alveolar epithelium, the pollutants can cross the alveoli-capillary barrier. There are various reports that demonstrate the damage caused to this organ by exposure to specific contaminants, such as vanadium that is part of the suspended particles.

In vivo, it has been reported that inhaled exposure to vanadium, mainly in the form of pentoxide induces histopathological changes in the lung, such as fibrosis [66], inflammation [30, 66, 67], hyperplasia and epithelial metaplasia [30, 67] and apoptotic cell death [68], among others.

Experimental evidence supports that exposure to V_2O_5 increases the production of ROS in lung cells. Wang et al. [68] reported increase in ROS production in mice bronchoalveolar lavage cells treated with a concentration of 10 μm of sodium metavanadate ($NaVO_3$), in a time-exposure dependent manner (3, 10, 30, and 60 minutes) through a spin trapping essay.

On the other hand, other evidence shows that exposure to V modifies in the lung glutathione concentrations, both in its oxidized (GSSG) and reduced (GSH) forms. It is known that oxidative stress results in the depletion of GSH and the increase in

GSS; so, the determination of their respective concentrations in blood and other tissues is considered a measure of intracellular oxidative stress [69].

Schuler et al. reported that in their inhalation model of V_2O_5 at exposure concentrations of 0.25, 1, and 4 mg/m^3, there was an increase in the levels of oxidized glutathione (GSSG) in lung tissue, with the consequent reduction in the ratio between reduced and oxidized glutathione (GSH/GSSG) concentrations [70]. Kulkarni and colleagues reported the same finding in relation to GSH concentration in lung tissue in a model of exposure to V_2O_5 nanoparticles [66]. In addition to this finding in the same study, the significant increase in MDA levels in plasma was identified. The MDA is a final product of lipid peroxidation.

Another biomarker of oxidative damage that has been identified is the 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxoGuo) in the DNA. Schuler demonstrated the increase in the formation of 8-oxoGuoin at exposure concentrations of 1 and 4 mg/m^3 of V_2O_5 in lung cells [70].

2.4.7 Nervous system

Neurotoxic metals as vanadium can induce oxidative damage in the brain and develop blood brain barrier disruption, neuropathology, and neuronal damage that can trigger central nervous system alterations as depression, increase in anger, fatigue, and tremors between other clinical features [71]. Also, a decrease in tyrosine hydroxylase and dopamine levels has been reported after vanadium exposure [72]. Chronical exposure to $NaVO_3$ can cause, in mice, metal accumulation in the olfactory bulb, brain stem, and cerebellum, as well as histopathological alterations like nuclear shrinkage in the prefrontal cortex and cell death of the hippocampal pyramidal cells and cerebellum Purkinje cells [71]. The accumulation of vanadium in the brain depends more on the exposure time than on the concentration of the metal. In fact, it is reported that disruption of ependymal cells is observed after long periods of vanadium inhalation [73].

Recently, behavioral alterations due to vanadium occupational exposure have been reported. Vanadium exposed workers exhibited poor performance in the simple reaction time, digit span memory, and Benton visual retention tests [74]. Memory loss in mice exposed to vanadium for 3 months was observed; nevertheless, in these animals, memory was recovered 9 months after vanadium was removal [75]. Increased incidence of Parkinson's disease is related to environmental metal exposure. It has been reported that vanadium pentoxide (V_2O_5) is neurotoxic to dopaminergic neurons via caspase-3-dependent PKCδ cleavage, so maybe vanadium can promote nigral dopaminergic degeneration [76].

2.5 Antioxidative action of carnosine and ascorbate

The cells exposed continuously to oxidative stress are not defenseless against free radicals. All aerobic organisms count with a series of mechanisms protecting them against oxidative damage; among them are antioxidant molecules which represent a first line of defense. If the antioxidant mechanisms fail, the cell uses others such as: transient cell arrest, biomolecular repair systems or apoptosis death processes [7].

An antioxidant is any substance that when is present in low concentrations, compared to the oxidizable substrate, decreases or prevents the substrate oxidation. Oxidizable substrates comprise everything that is found in living tissues including proteins, lipids, carbohydrates, and nucleic acids [77].

Cells use a series of antioxidant compounds that react directly with oxidizing agents, functioning as "sweepers" or chemical shields [7]; these molecules have enzymatic or non-enzymatic actions. Non-enzymatic antioxidants carry out

the reduction of free radicals through electron donation, thus avoiding oxidative reactions. Glutathione (GSH), alpha-tocopherol (vitamin E), ascorbic acid (vitamin C), carnosine, bilirubin, and uric acid are the main molecules performing this function.

Ascorbate is an important water-soluble antioxidant in biological fluids, because it eliminates reactive oxygen species and radicals such as: alkoxy, hydroxyl, peroxyl, and hydroperoxyl radicals, singlet oxygen, superoxide anion, and ozone. It also eliminates reactive species and radicals derived from nitrogen and chlorine and even radicals that come from other antioxidants [78].

In general, a large number of studies have been carried out to show the beneficial effects of ascorbate. Evidence indicates that supplementation with this compound protects against lipid oxidation *in vivo*, particularly in individuals exposed to exacerbated conditions of oxidative stress, such as smokers [79].

Epidemiological studies of treatment with this antioxidant have shown consistently favorable effects in patients with cardiovascular disease or coronary risk. In addition, it has been suggested that the increase in ascorbate consumption significantly decreases the incidence and mortality from cardiovascular diseases. Even in pathologies related to free radicals and the inability of the organism defenses against them, as is the case of cancer, epidemiological studies show that increased consumption of ascorbate decreases the incidence and mortality from cancer [79].

Experimental evidence indicates that ascorbic acid works as an antidote against acute vanadium poisoning. In mice, Jones and Basinger [80] examined several compounds and concluded that ascorbate was the most promising for human use.

Domingo et al. [81] administered $NaVO_3$ to mice intraperitoneally and observed, as did Jones and Basinger, that ascorbate proved to be the most effective antidote against vanadium poisoning. In another study, Domingo et al. [82] showed that ascorbate stimulates urinary excretion of vanadium when mice are injected intramuscularly with $VOSO_4$.

Another water-soluble antioxidant is carnosine which is a dipeptide composed of β-alanine and L-histidine; it is found naturally in many mammalian species, mainly in the skeletal muscle. It is estimated that 99% of the carnosine in the organism is found in muscular tissue [83].

It has been reported that carnosine may form complexes with transition metals and has antioxidant activity, which implies mechanisms such as chelation of metals, scavenging of ROS, and peroxyl radicals [83].

The antioxidant efficiency of carnosine in the nervous system, when mice are exposed to vanadium inhalation was successfully tested by our group. It was observed that in those groups with carnosine treatment, a larger size granulose cells with a greater number of dendritic spines, and in general less adverse ultrastructural morphological changes, as well as less lipid peroxidation were observed [84].

3. Conclusions

Air pollution has been continuously mentioned as one of the problems that decrease the quality and life expectancy of all living organisms, included humankind. It is true that not all the sources of pollution are from anthropological origin; however, a great deal of it are generated by humans and can be prevented or controlled by those who generate it.

The use of fossil fuels as the quasi unique source of energy and limited use of other sources of energy will maintain the air pollutant levels high enough to keep its deleterious health effects.

As it is revised in this chapter, metals are one of the air pollutants that enter through the respiratory tract, reaching by the systemic circulation every cell in living organisms. Vanadium is one of the elements adhered to PM which results from the incomplete combustion of fossil fuels. PM generates ROS, mainly those that contains transition metals (e.g., Fe, V, and Mn).

Reported previously in this chapter, one of the main toxic mechanisms of metals is oxidative stress which affects all biomolecules. DNA oxidative damage may conduct the cell to genotoxic and mutagenic changes and further to cell death or cancer.

When proteins are oxidized: cell structure, cell signaling modification, and/or disruption of cellular enzymatic processes could be noticed. The reactive molecules which results from these interactions with proteins and ROS may interplay with specific peptide residues such as: lysines, arginines, histidines, and cysteines. The result of these actions causes the formation of reactive carbonyls and protein carbonylation, and its accumulations have been related with chronic diseases and aging.

If lipids are in contact with ROS, peroxidation occurs producing MDA, a biomarker of oxidative stress that could interact with proteins forming protein adducts and inactivating the protein. Another lipid peroxidation product is 4-HNE with cytotoxic effects and the induction of pro-inflammatory cytokines, which could result in cellular dysfunction and death [85].

If the sources of V or other pollutants are not reduced and the oxidative insults prevail, we can supplement our system with antioxidants such as vitamin C. This water-soluble molecule is not synthesized by humans, and its supplementation is obtained by different dietary sources such as fruits and vegetables or by vitamin C supplements. One of the benefits of vitamin C is its antioxidant action, scavenging ROS and NOS species. In addition, it helps to regenerate alpha-tocopherol and coenzyme Q; also, vitamin C inhibits NAD(P)H oxidase decreasing ROS formation [86]. Another less known endogenous and exogenous antioxidant is carnosine that in our laboratory showed promising antioxidant effects in the nervous system [84].

The systems and organs affected by the oxidative potential of vanadium and the protective effect of antioxidants are summarized in **Figure 2**.

While humankind decide to work together in order to find a common solution for controlling air pollution, scientist should be working in finding more and better

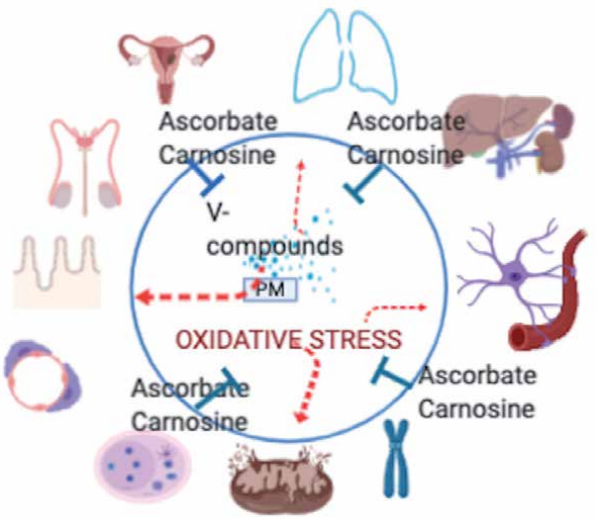

Figure 2.
Oxidative stress by vanadium and antioxidants protective effects (this figure was created by Biorender software in www.biorender.com).

antioxidants to prevent and ameliorate the effects that metals, such as V adhered to PM, have on living organisms, that meanwhile might reduce oxidative stress, its injurious effects and improves the quality of life on the planet.

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Chapter 7

The *w*/*w*$^+$ Somatic Mutation and Recombination Test (SMART) of *Drosophila melanogaster* for Detecting Antigenotoxic Activity

Isabel Gaivão, João Ferreira and Luisa María Sierra

Abstract

Genotoxicological studies are emerging as fundamental for knowing the hazards to our genome, to our health. *Drosophila melanogaster* is one of the preferable organisms for toxicological research considering its metabolic similarities (viz. on dietary input, xenobiotic metabolizing system, antioxidant enzymes and DNA repair systems) to mammals. Accordingly, somatic mutation and recombination tests (SMARTs) of *D. melanogaster* are fast and low-cost in vivo assays that have shown solid results evaluating genotoxicity. The *w*/*w*$^+$ SMART uses the *white* (*w*) gene as a recessive marker to monitor the presence of mutant ommatidia (eye units), indicating the occurrence of point mutations, deletions, mitotic recombination or/and nondisjunction. Additionally, several studies used SMARTs to assess antigenotoxicity, with some using the *w*/*w*$^+$ SMART. We reviewed the state of the art of the *w*/*w*$^+$ SMART used for antigenotoxicity analysis, focusing on published results, aiming to contribute to the conception of a reliable protocol in antigenotoxicity. As such, genotoxic agents with known action mechanisms, as streptonigrin (oxidative stress inducer), were used as a genotoxic insult for proving the antigenotoxic effects of natural substances (e.g. seaweeds), demonstrating the presence of antimutagens in their composition. These antigenotoxicity studies are crucial for promoting preventive measures against environmental genotoxics that affect humans daily.

Keywords: genotoxicity test, *w*/*w*$^+$ SMART, eye-spot test, *Drosophila melanogaster*, streptonigrin, genotoxic agent, oxidative stress, DNA damage, ROS inhibition, antigenotoxicity, antimutagens, dietary antioxidants

1. Introduction

The environmental emergency is largely related to environmental toxicology. Each day, new molecules are synthesized, or natural molecules are intensively produced that enter in ecosystems and affect them at all levels. Nowadays there are circulating in living organisms thousands of substances that did not exist 100 years ago, with somewhat unpredictable consequences. As such, more than 159 million chemical substances are registered in the Chemical Abstracts Service (CAS), with

approximately 4000 new substances being registered daily [1]. As a controlling measure, the EU Commission created, in 2004, a network (NORMAN network) of laboratories, research centres and organizations for monitoring the emerging environmental substances [1].

Environmental toxicology encompasses exposure to toxic substances whether through the air we breathe, the food we eat, the water we drink and the clothes we wear or through the skin, cosmetics, etc. There is also radiation exposure, which also has harmful effects, and is much more problematic today than some years ago. The planet is poisoned, affecting the air, the water, the soil and the food we produce, which causes serious problems to human health and ecosystems. It is hoped that worldwide awareness of this reality will be achieved, and the focus of humanity's greatest concerns will be on the cleansing of the planet by eliminating or at least greatly reducing the produced toxic agents.

This whole problem greatly affects DNA, causing DNA damage (genotoxicity), affecting DNA repair mechanisms and causing mutations when damage is not properly repaired. In the short term, this genome instability leads to diseases such as cancer, degenerative diseases, fertility decrease and other problems. In the long term, we may see the emergence of new diseases due to new mutations in the germ line, which, if recessive, may take several generations until there is a chance of homozygosis, where rare diseases may arise. All combined may affect the life expectancy of several species, causing an environmental collapse. Preventive strategies are indispensable to reduce the heavy burden on national healthcare systems and families. The most effective is a healthy lifestyle including diet, as an antigenotoxic diet reduces DNA damage and all the associated diseases. Antigenotoxic activities include inactivation of genotoxic compounds, by several mechanisms and increasing repair capacity, decreasing the effectiveness of a genotoxic. While DNA damage is clearly implicated as the initiating event in most cancers, the link is not a simple one. Most damage is removed by repair enzymes before it can interfere with the process of DNA replication and introduce mutations. Given a carcinogenic exposure, the individual variation in the capacity for DNA repair is therefore likely to be an important factor in determining cancer risk.

Over the years, many investigations in DNA damage and DNA repair mechanisms were made, in vitro and in vivo, aiming to know our environment and thus identifying the harmful compounds to our genome, to our health, leading to preventive actions such as prohibiting the commercialization of certain drugs, construction materials, foods and drinks. Genotoxicological studies using cell cultures and animals are essential for increasing human's wellbeing, since they display solid results in showing the genotoxicity of compounds and should be standardized (with optimal test conditions) for increasing their reproducibility and precision.

2. *Drosophila melanogaster* in toxicological research

Drosophila melanogaster is currently being used as one of the preferable organisms for toxicological research [2]. According to current knowledge, the use of *D. melanogaster* as a model organism respects the principles of animal welfare (3Rs), since ethical matters do not urge when using this organism [2, 3]. Considering the metabolic pathways responsible for dietary input (including nutrient uptake, digestion, absorption, storage and metabolism) [4], the xenobiotic metabolizing system, the antioxidant enzymes and the DNA repair systems of *D. melanogaster*, which are analogous to those of mammals, *D. melanogaster* emerges as an optimal replacer of higher animals in toxicological studies [2, 5]. Furthermore, contrasting

with in vitro methods, *D. melanogaster* has the advantage of enabling a more solid extrapolation at the organism level [3].

D. melanogaster exposure to toxic agents leads to the alteration of simple life traits, which are perturbed negatively, such as development time, number of eclosed individuals, sex ratio, adult body size, fertility and others [6, 7]. These life traits can be assessed as a way of measuring the toxicological effects of a given drug, food, drink and so on. However, as science progresses and hazards are targeted in a more specific way, genotoxicological studies with *D. melanogaster* were developed, aiming to identify environmental hazards inducing damages to genome, *i.e.* genotoxic agents. In this way, genotoxicological studies with *D. melanogaster* deal with the assessment of changes in genetic material through various assays, such as germ line mutation assays, somatic mutation assays, the chromosomal aberration assay, the micronucleus test, the comet assay and DNA sequence-based assays, among others. In particular, somatic mutation and recombination tests (SMARTs) have proven to be a good tool for detecting a broad range of genetic alterations quickly and inexpensively [2, 8].

2.1 Somatic mutation and recombination tests of *D. melanogaster*

The somatic mutation and recombination tests of *D. melanogaster* have shown excellent results in assessing the genotoxicity of several and diversified compounds in somatic cells. Originally, in the 1980s, the SMART could be performed by four different assays, but only two of them made it through to the present day: the wing-spot test and the eye-spot test (or *w*/*w*$^+$ SMART) [9]. The wing-spot test was firstly described by Graf and Würgler [10] and the *w*/*w*$^+$ SMART by Würgler and Vogel [11], with both showing high values of sensitivity, specificity and accuracy.

Briefly, in the late embryogenesis, larval structures are set, and groups of diploid cells of undifferentiated epithelium, imaginal discs, are formed in the embryo [12]. Then, upon the ending of the larval stages, pupa emerges, and metamorphosis takes place upon systemic hormonal regulation, with the histolysis of the larval organs and differentiation of the imaginal discs into adult structures [13, 14]. Accordingly, the exposure of imaginal discs to genotoxic agents may lead to genetic alterations (the product of DNA damage) capable of being transmitted to daughter cells upon mitosis. These genetic alterations can be phenotypically manifested in the adults in structures such as the wings and the eyes, which can be assessed according to the wing-spot test and the eye-spot test, respectively. The loss of heterozygosity (LOH) for specific genetic markers in heterozygous individuals allows the quantification of DNA damage/level of genotoxicity in the adult tissues by visual scoring [9, 15].

Between the two types of SMART currently used, from the practical point of view, the *w*/*w*$^+$ SMART can be assayed with six different strains, as firstly shown by Vogel and Nivard [16], contrasting with only two strains available for the wing-spot one; in the *w*/*w*$^+$ SMART, a standardized genotoxic agent, inducing a high genotoxicity without toxic effects, streptonigrin (further focused on the chapter) [17], is available and has proved its effectiveness. Nevertheless, since the wing-spot test allows the visual scoring of wings over time, considering that wings are mounted/preserved on slides, opposite from what happens in the *w*/*w*$^+$ SMART, where eyes have to be analysed quickly since no preserving actions are available (time limited scoring), a greater number of studies have been performed using the wing-spot test (**Table 1**). Henceforward, as a measure of further exploring the potential of this test and increasing its number of studies, the *w*/*w*$^+$ SMART will be focused.

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
Abraham [18]	Wing-spot	Cyclophosphamide (CPH) Diethylnitrosamine (DEN) Mitomycin C (MMC) Procarbazine (PRO) Urethane (URE)	Coffee	+ + + − +
Alaraby et al. [19]	Wing-spot	Potassium dichromate (PD)	CeO_2 NPs Cerium sulphate	+ +
Alaraby et al. [20]	Wing-spot	Potassium dichromate (PD) Ethyl methanesulfonate (EMS) Potassium dichromate (PD) Ethyl methanesulfonate (EMS)	CuO NPs Copper oxide	+ +
Amkiss et al. [21]	Eye-spot	Methyl methanesulfonate (MMS)	Fennel plant fruit extracts	+
Anter et al. [22]	Wing-spot	Hydrogen peroxide	Virgin Olive oil Triolein Tyrosol Squalene	+ + + +
Anter et al. [23]	Wing-spot	Hydrogen peroxide	Red table grapes	+
Anter et al. [24]	Wing-spot	Hydrogen peroxide	Phenols: apigenin, bisabolol, protocatechuic acid	+ + +
Aydemir et al. [25]	Wing-spot	Fotemustine	Amifostine	+
Cápiro et al. [26]	Eye-spot	Methyl methanesulfonate (MMS) Ethylnitrosourea (ENU) Juglone (JG) Dimethylbenz(a)anthracene (DMBA)	*Cymbopogon citratus*	+ + + +
Demir and Marcos [27]	Wing-spot	Potassium dichromate	Boron nitride nanotubes	+
De Rezende et al. [28]	Wing-spot	Doxorubicin (DXR)	Grape seed proanthocyanidins	+
De Rezende et al. [29]	Wing-spot	Doxorubicin (DXR)	Dibenzylbutyrolactolic lignan(−)-cubebin	+/−
Drosopoulou et al. [30]	Wing-spot	Mitomycin C (MMC)	Chios mastic products: verbenone α-terpineol linalool trans-pinocarveol	+ + + −
El Hamss et al. [31]	Wing-spot	Urethane (URE)	Turmeric	+
Fernandes et al. [32]	Wing-spot	Doxorubicin (DXR) Benzo(a)pyrene (B(a)P)	Vitexin	+ +
Fernandez-Bedmar and Alonso-Moraga [33]	Wing-spot	Hydrogen peroxide	Green sweet pepper Red sweet pepper Green hot pepper Red hot pepper Capsaicin Capsanthin Lutein	+ + − + + + +

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
Fernández-Bedmar et al. [34]	Wing-spot	Hydrogen peroxide	Citrus juices Hesperidin Limonene	+ + +
Fernandez-Bedmar et al. [35]	Wing-spot	Hydrogen peroxide	Tomato Lycopene	+ +
Fernández-Bedmar et al. [36]	Wing-spot	Hydrogen peroxide	Garlic Onion Diallyl disulphide Dipropyl disulphide	+ + + +
Ferreira et al. [3]	Eye-spot	Streptonigrin (SN)	*Grateloupia turuturu* *Porphyra umbilicalis*	+ +
Graf et al. [37]	Wing-spot	Urethane (URE) Methyl urea + sodium nitrite	Instant coffee Ascorbic acid Catechin	+ + +
Guterres et al. [38]	Wing-spot	Doxorubicin (DXR)	*Momordica charantia*: aerial parts Fruit	– +
Idaomar et al. [39]	Wing-spot	Urethane (URE)	Essential oils from: *Helichrysum italicum*, *Ledum groenlandicum*, *Ravensara aromatica*	+ + +
Kylyc and Yesilada [40]	Wing-spot	Mitomycin C (MMC)	Dried mycelia from: *Trametes versicolor* *Pleurotus ostreatus*	+ +
Laohavechvanich et al. [41]	Wing-spot	Urethane (URE)	Bird pepper Red chili spur pepper Green bell pepper Green sweet pepper	+ + + +
Lozano-Baena et al. [42]	Wing-spot	Hydrogen peroxide	*Brassica carinata* Sinigrin	+ +
Marques et al. [43]	Eye-spot	Streptonigrin (SN)	*Ulva rigida* *Fucus vesiculosus* *Gracilaria* species	+ + +
Martinez-Valdivieso et al. [44]	Wing-spot	Hydrogen peroxide	Lutein β-Carotene Zeaxanthin Dehydroascorbic acid Yellow zucchini Light green zucchini	+ + + + + +
Mateo-Fernandez et al. [45]	Wing-spot	Hydrogen peroxide	Caramel color class IV	+
Merinas-Amo et al. [46]	Wing-spot	Hydrogen peroxide	Choline	+
Mezzoug et al. [47]	Wing-spot	Urethane (URE)	*Origanum compactum* essential oil	+
Niikawa et al. [48]	Wing-spot	Mitomycin C (MMC)	Salicylic acid Salicyluric acid Gentisic acid Gentisuric acid 2,3-Dihydroxybenzoic acid	– + + + +

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
Niikawa et al. [49]	Wing-spot	Mitomycin C (MMC)	Salicylic acid	–
			Salicyluric acid	+
			Gentisic acid	+
			Gentisuric acid	+
			2,3-Dihydroxybenzoic acid	+
Oliveira et al. [50]	Wing-spot	Doxorubicin (DXR)	Metformin	+
Orsolin et al. [51]	Wing-spot	Doxorubicin (DXR)	Simvastatin	+
Pádua et al. [52]	Wing-spot	Mitomycin C (MMC)	*Terminalia*	+
		Ethyl methanesulfonate (EMS)	*actinophylla* extracts	+
Patenkovic et al. [53]	Wing-spot	Methyl methanesulfonate (MMS)	Sage tea	+
Patenkovic et al. [54]	Wing-spot	Methyl methanesulfonate (MMS)	Gentian tea	–
Prakash et al. [55]	Wing-spot	Ethyl methanesulfonate (EMS)	Caffeine	+
Prakash et al. [56]	Wing-spot	Methyl methanesulfonate (MMS)	*Dioscorea pentaphylla*	+
Rizki et al. [57]	Wing-spot	Potassium dichromate (PD)	Sodium selenite	+
Romero-Jiménez et al. [58]	Wing-spot	Hydrogen peroxide	*Matricaria chamomilla*	+
			Tilia cordata	+
			Mentha piperita	+
			Mentha pulegium	+
			Uncaria tomentosa	+
			Valeriana officinalis	+
Sarıkaya et al. [59]	Wing-spot	Ethyl methanesulfonate (EMS)	Boron	+
Savić et al. [60]	Wing-spot	Methyl methanesulfonate (MMS)	Royal Sun Agaricus extract	–
Sukprasansap et al. [61]	Wing-spot	Urethane (URE)	Eggplants	+
Taira et al. [62]	Wing-spot	2-AAF	*Agrocybe cylindracea*	+
		Aflatoxin B1	*Lentinula edodes*	+
		DMBA	*Pleurotus ostreatus*	+
		IQ		+
		MeIQx		+
		MNU		+
		NDMA		+
		4NQO		+
		2-AAF		-
		Aflatoxin B1		+
		DMBA		–
		IQ		–
		MeIQx		–
		MNU		+
		NDMA		+
		4NQO		+
		2-AAF		+
		Aflatoxin B1		+
		DMBA		+
		IQ		+
		MeIQx		+
		MNU		+

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
		NDMA		+
		4NQO		+
Tasset-Cuevas et al. [63]	Wing-spot	Hydrogen peroxide	Borage seed oil	+
			Gamma linolenic acid	+
Toyoshima et al. [64]	Wing-spot	Sun and UV light	Sunscreens:	+
			SPF 20	+
			SPF 40	+
			SPF 60	
Valadares et al. [65]	Wing-spot	Doxorubicin (DXR)	Propolis (water extracts)	+
Valente et al. [66]	Eye-spot	Streptonigrin (SN)	Thalassotherapy products	+

The type of test, wing- or eye-spot, the used genotoxic agents, as well as the information about the antigenotoxic potential of the tested substances (response: + antigenotoxic activity; − no antigenotoxic activity or synergistic genotoxic activity) is presented.

Table 1.
Published studies focusing the antigenotoxic evaluation of several types of chemicals, nanoparticles and plants/seaweeds/seeds/oils using somatic mutation and recombination tests (SMARTs).

3. *w*/*w*$^+$ SMART (eye-spot test)

D. melanogaster presents two symmetrically positioned eyes in its head. Each eye consists of repeated hexagonal arrays of approximately 750–800 ommatidia (eye units formed upon differentiation of imaginal discs), homogenous in size and regularly spaced, with each ommatidium being constituted by 14 cells (8 photoreceptor cells, 4 cone cells and 2 primary pigment cells) [67]. Between each two ommatidia, six secondary pigment cells, three tertiary pigment cells and three mechanosensory bristle complexes are present [67]. The adult eye of *D. melanogaster* is particularly used in toxicological assays since subtle defects in ommatidia development are amplified, by mitosis, several hundred times in the eye [68]. Therefore, it is quite simple to detect genetic alterations changing its pigmentation.

The basis of the *w*/*w*$^+$ SMART is the *white* (*w*) gene located at the position 1.5 of the X chromosome. This gene is used as a recessive genetic marker to monitor the presence of mutant ommatidia/spots, indicating the occurrence of LOH by deletions, point mutations, mitotic/somatic recombination (the most frequent) or/and nondisjunction (chromosome losses) in somatic cells (**Figures 1** and **2**) [9, 16]. These genetic events are known to display a significant role in the induction of carcinogenesis [69]. Accordingly, when wild-type females (*w*$^+$/*w*$^+$; red eyes) are crossed with white-eyed males (*w*/Y; eyes without pigmentation), or vice versa (*w*/*w* with *w*$^+$/Y), a heterozygous offspring is developed for females (*w*$^+$/*w*; red eyes). However, if the offspring is exposed to genotoxic agents in its development phase, the presence of white/mutant phenotype spots in the red eyes may occur (**Figures 1** and **2**). In addition, when crossing wild-type females with white-eyed males, males' eyes can also be analysed, although somatic recombination should not be considered in this case [9]. The difference between females and males scoring will provide quantitative information on somatic recombination [9].

Moreover, Vogel and Nivard [69, 70] designed a more refined, as well as time-consuming, version of the *w*/*w*$^+$ SMART, which allows the detection of chromosomal aberrations in late larval stages. However, and according to Marcos and Sierra [9], the ratio of results obtained/time consumption is low in comparison with the

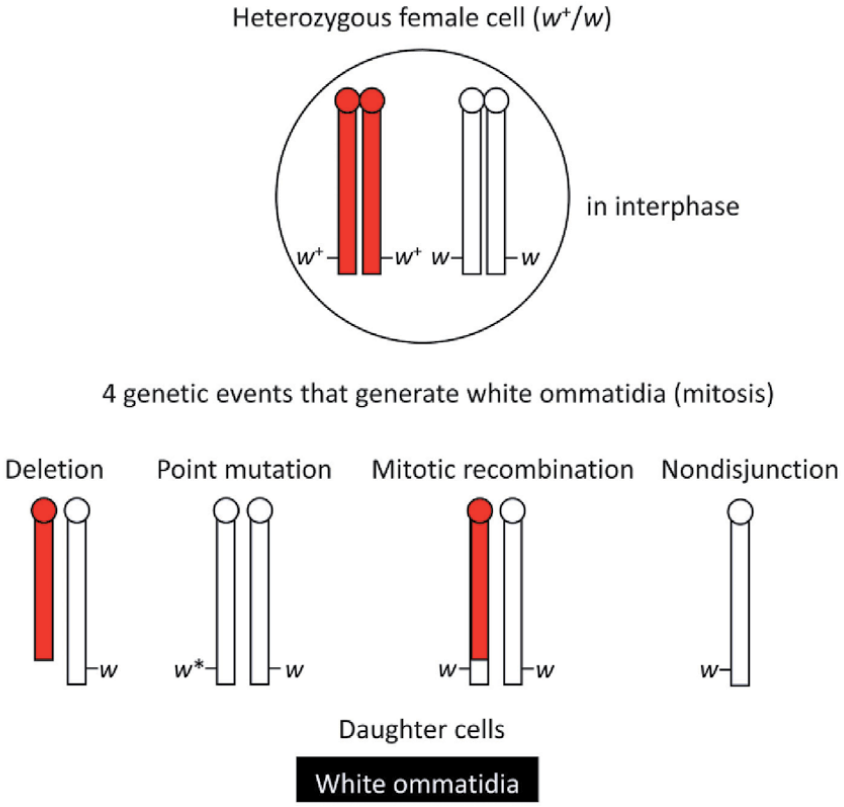

Figure 1.
Scheme of the possible four types of genetic alterations that generate white ommatidia in a heterozygous D. melanogaster *female for the white (w) gene. In the scheme, the heterozygous female cell has two X chromosomes with two chromatids each (duplicated DNA in interphase) and daughter cells have two X chromosomes but only one chromatid each (except for nondisjunction). The X chromosomes in red carry the w⁺ allele (dominant) and those in white carry the w allele (recessive), however there are a few exceptions that will be described below. The position of the alleles in the X chromosomes is represented in a purely illustrative, non-exact way. w* is a mutated wild-type expressing white phenotype. In the development phase of a heterozygous female for the w gene (w⁺/w), genetic alterations may be induced in the imaginal discs and, upon cell division, daughter cells with mutant/white phenotype ommatidia may appear. The genetic alterations that cause mutant phenotypes are: deletion in one X chromosome including the white locus (in the wild-type allele); point mutation in the wild-type allele by substitution, insertion, or deletion; mitotic recombination between chromatids of the homologous X chromosomes, that replaces the wild-type locus by a mutant locus; nondisjunction, that causes the loss of the chromosome with the wild-type allele.*

original version of the assay, making it less efficient in the laboratorial routine. Thus, the original version of the assay continues to be the main choice when performing *w*/*w*⁺ SMART.

3.1 Antigenotoxicity with *w*/*w*⁺ SMART

w/*w*⁺ SMART was, in its original concept, used for the genotoxicological evaluation of several chemical agents, directed to unveiling the action mechanisms behind their genotoxic activities [17, 71–73]. As such, alkylating agents, such as methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS) and ethylnitrosourea (ENU), are between the chemicals that induce a great number of mutant ommatidia in *D. melanogaster* [72]. Even so, and considering the study from Gaivão and Sierra [17], a quinone-based antibiotic, streptonigrin (SN), showed its potential to induce a great level of genotoxicity (increased number of mutant

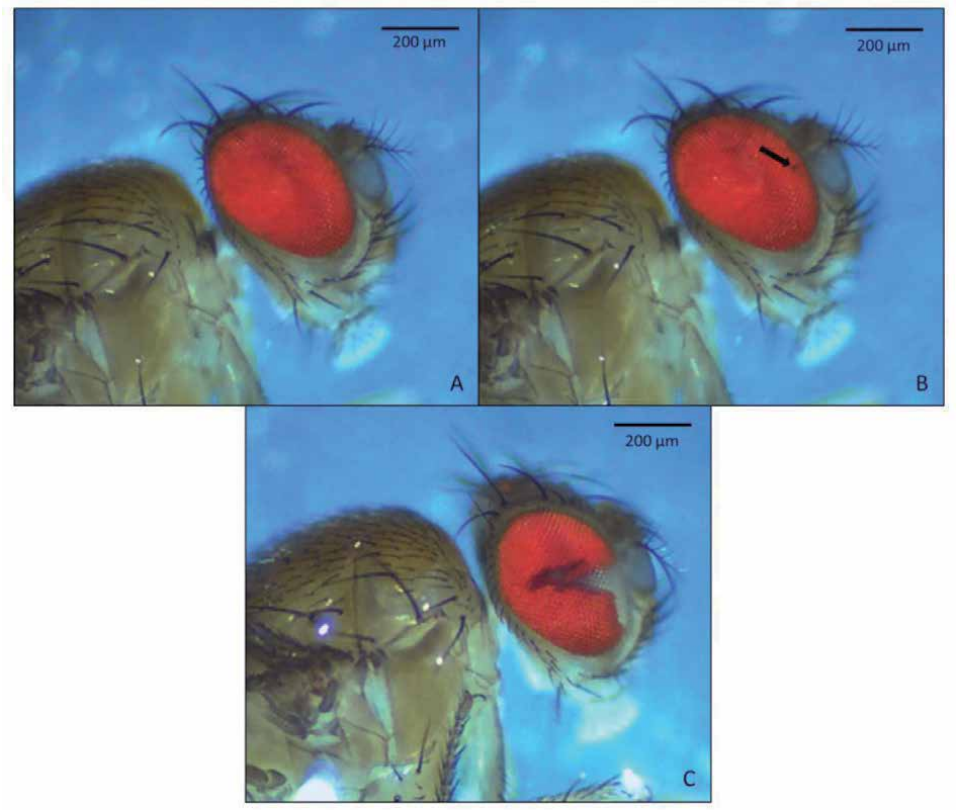

Figure 2.
Wild-type eyes of D. melanogaster *(females) at the stereoscopic microscope (80× magnification). (A) An eye without mutant spots, (B) an eye with a dark spot affecting one to two ommatidium(a) (marked by a black arrow) and (C) an eye with a spot affecting innumerable ommatidia. White mutant spots appear as black when surrounded by pigmented/red ommatidia.*

ommatidia) without toxic effects (at 20 μM) in the *w*/*w*$^+$ SMART, making it a suitable genotoxic insult for this assay. SN, in the presence of certain metal cations (Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Cd^{2+} and/or Au^{2+}), binds to DNA establishing SN-metal-DNA complexes, known as DNA adducts [74–76] (**Figure 3**). Upon the binding, the quinone reduces, via one or two e^- (NADH as a cofactor), producing a semiquinone or a hydroquinone, respectively. Semiquinone reacting with O_2 leads to the production of O_2^- and quinone regeneration. Hydroquinone can lead to the production of H_2O_2, while quinone is regenerated (**Figure 3**). In consequence, OH can be produced by the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow OH + OH^- + Fe^{3+}$) and by the Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow OH + OH^- + O_2$), leading to oxidative stress [74–76]. The production of reactive oxygen species (ROS), and the prolonged SN linkage to DNA, can lead to the inhibition of DNA (and RNA) synthesis, induce unscheduled DNA synthesis, promote DNA strand breaks as well as inhibit topoisomerase II [77]. Chromosomal aberrations may occur upon mutagenic events, creating genomic instability that can culminate into carcinogenic events [76] (**Figure 3**).

Among the processes related to genotoxicity, with an increased relevance in the last years, the analysis of antigenotoxicity is probably the most important one. The search for antigenotoxic agents that could prevent or counteract the harmful consequences of the exposure to DNA damaging agents has increased exponentially lately [78–80]. Since most of the possible antigenotoxic agents are components of natural products that could be included in the diet, the analysis of their properties

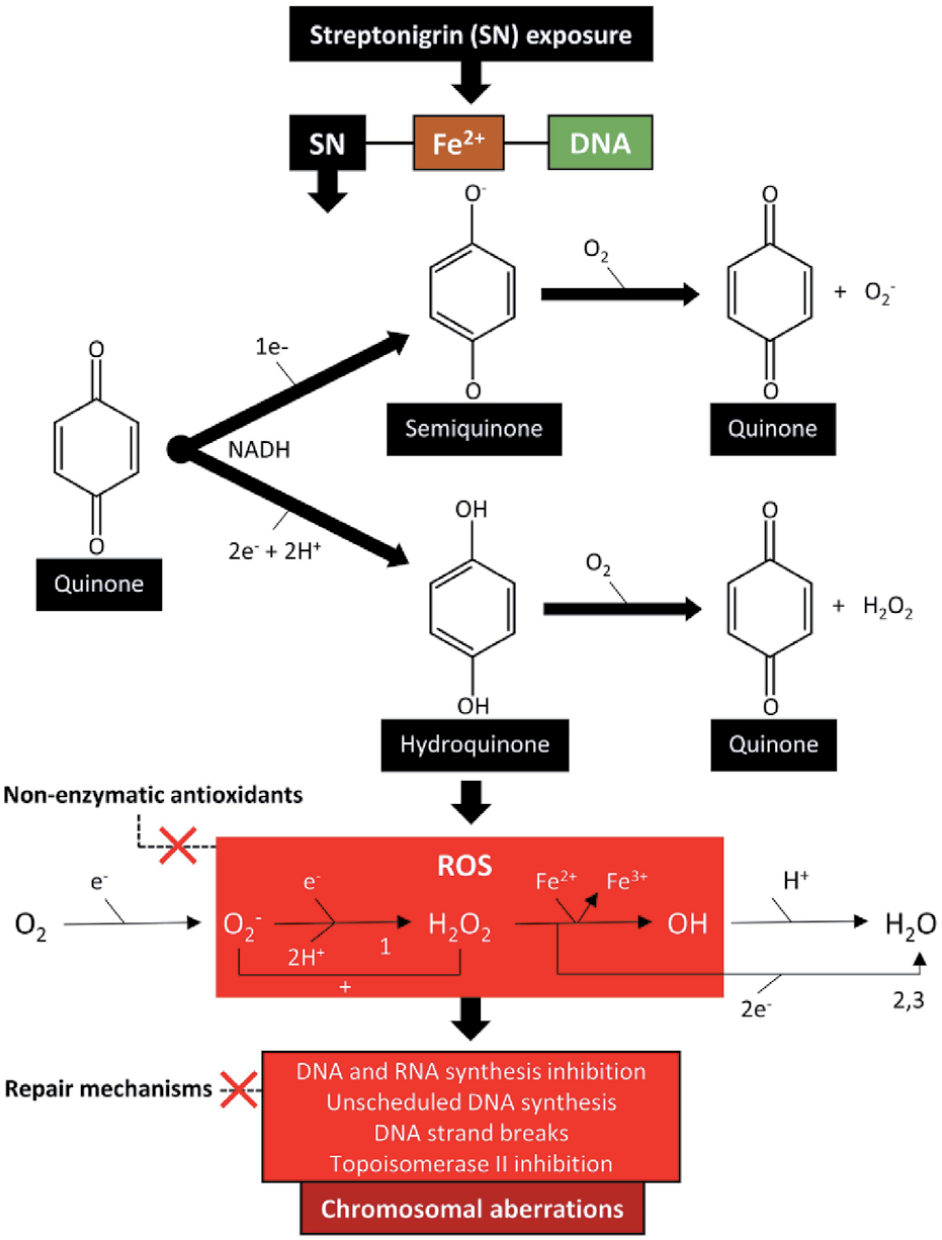

Figure 3.
Simplistic scheme of the genotoxic activity of streptonigrin (SN) on an animal cell. Cell exposure to SN leads to the formation of DNA adducts [SN + metal cation (such as Fe^{2+}) + DNA]. SN's quinone groups are reduced (NADH as a cofactor) to semiquinone and hydroquinone that, in the presence of O_2, lead to the formation of O_2^- and H_2O_2, respectively, both with quinone regeneration (vicious cycle). Thus, by chemical reactions (such as the Fenton and Haber-Weiss ones), OH is produced, the most severe reactive oxidative species (ROS). In this case, the antioxidants (endogenous enzymatic and non-enzymatic, and dietary inputs) are not capable of avoiding excessive ROS formation and progression, as well as communicating to repair mechanisms for repairing the induced genetic damages that may lead to chromosomal aberrations. (1) Superoxide dismutase (SOD); (2) catalase (CAT); (3) glutathione peroxidases.

should be performed in in vivo experiments. As so, *Drosophila* fulfils all the requirements for this analysis, specifically when using SMARTs. In fact, there are numerous published studies using *D. melanogaster* in antigenotoxicity analyses, and most of them are using SMARTs, especially with the wing-spot test (**Table 1**).

Focusing on the *w*/*w*$^+$ SMART performed for antigenotoxicity testing, there are a few studies evaluating the antigenotoxic potential of lemongrass extracts [26]; fennel plant fruit extracts [21]; red, green and brown seaweeds [3, 43]; and thalassotherapy products (containing seaweeds) [66].

Ferreira and Marques [3] and Marques and Ferreira [43] studied the exposure of *D. melanogaster* [*Oregon-K* (*OK*) strain] to a chronic treatment (from egg to adult eclosion) with media (Formula 4-24® Instant Drosophila Medium) supplemented with red, green or brown seaweeds and SN (at 20 μM). Reductions in the number of mutant ommatidia were shown in individuals cotreated with seaweed and SN in relation to the positive control. Thus, protective properties of seaweeds were exerted against the genotoxic insult of SN, demonstrating antigenotoxic potential. Even more, some species displayed antigenotoxic effects against the spontaneous genotoxicity (without SN insult) of *D. melanogaster*. The authors also refer the possible phytochemicals acting as antimutagens that include vitamins, phenolic compounds, pigments and polysaccharides. These phytochemicals, which may promote their action in a synergetic way, may inhibit ROS triggered by SN activity, acting as dietary antioxidants [3] (**Figure 3**). Their mechanisms of action may include ROS scavenging, donation of electrons and/or protons to endogenous enzymatic and/or non-enzymatic antioxidants for converting ROS to H_2O and/or chelating metal ions responsible for producing OH (Fenton reaction inhibition) [34, 81]. In line, using the same conditions, Valente and Borges [66] showed the antigenotoxicity of thalassotherapy products (with seaweeds) against SN. Once more, the potential of seaweeds as dietary antioxidants/antimutagens, as well as the potential of SN as an optimal inducer of chromosomal aberrations quantifiable by the SMART, was demonstrated. Longevity-promoting properties were also displayed upon seaweed supplementation which, according to free radical and mitochondrial theories of ageing, may be a collateral effect of the dietary antioxidants that modulate the enzymatic antioxidants and exert direct antioxidant-scavenging actions [3, 66].

MMS (at 1 mM) was used as a genotoxic insult against a fennel plant fruit aqueous extract [21]. The positive control showed a great number of induced mutant ommatidia, proving the results from Vogel and Nivard [72], and the fennel extract showed antigenotoxic activity against MMS. According to the authors, and considering the mutagenic activity of MMS, an alkylating agent, consisting of direct interactions with DNA bases that induce mutagenic events, fennel may possess antimutagens that interact directly with the methyl radical groups of MMS and inactivate them in such a manner that they cannot bind to DNA as effectively to induce their mutagenic activity. The antimutagenic properties displayed by fennel may be related to components of its essential oil [21]. In a similar way, Cápiro and Sánchez-Lamar [26] demonstrated the antigenotoxic potential of lemongrass decoction extracts against different genotoxics, MMS, ENU, juglone (JG) and dimethylbenz(a)anthracene (DMBA), that exhibit different mechanisms of action. According to the authors, the lemongrass extract modulated the genotoxic action of the alkylating agents MMS and ENU by interacting with them directly or/and with their mutagenic derivatives. Regarding JG, a naphthoquinone that induces ROS production in an analogous way to SN, damages were reduced upon exposure to the decoction extract by probably inhibiting ROS production, by sequestrating/inhibiting ROS activity or/and activating intracellular defence mechanisms. For DMBA, as it needs metabolic activation by microsomal enzymes, the extract may have interfered with the microsomal enzymatic system for avoiding DMBA activation. Overall, lemongrass extract acted as an antimutagen in the protection of DNA.

In fact, SMART can be assayed using different test conditions, including the *D. melanogaster* strain (OK strain has potential for genotoxicity testing; presents high

susceptibility to ROS, mainly due to a low activity of antioxidant enzymes, being more sensitive to increase its antioxidant status upon intake of dietary antioxidants [3, 73]), treatment method (chronic or acute and pre-, co- and post-treatments), genotoxic agent (should always be chosen among those with a known mechanism of action; an example is SN) and sample size. For more details on the methodological approaches of SMARTs, see the protocol from Marcos and Sierra [9].

4. Conclusions

In vitro and especially in vivo genotoxicity testing of substances such as foods, drinks, drugs and herbicides is fundamental for increasing humans' knowledge on the hazards that we may be exposed to. In this way, upon the identification of a substance/compound as genotoxic, priorities should be focused on avoiding this genotoxic or, at least, when the exposure is unavoidable, preventing our metabolism from damages to DNA that can culminate in mutagenic events and, in a later stage, on carcinogenesis. Upon in vitro testing, in vivo genotoxicological assays, such as w/w^+ SMART in *D. melanogaster*, are great tools for evaluating the antigenotoxic potential of a given substance/compound, considering optimal test conditions. The ultimate objective of these tests is to promote the dietary intake of antimutagens, since they are essential for reinforcing our metabolic defences towards genotoxic events, especially the ones that may be produced by strong exogenous agents. Foods, teas, nutraceuticals and others who are richly composed of dietary antimutagens should be of daily intake, considering that there is an increasing threat of new chemical substances with genotoxic potential every day.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 8

Current Trends and Future Perspectives of Antimutagenic Agents

Adel M. AbdelHakem and El-Shimaa M.N. Abdelhafez

Abstract

Mutation is the process leading to heritable changes in DNA caused mainly by internal and external factors. Recently, studies on mutagenic agents have been increased due to increasing in mutation-related disease. The antimutagenic effect is desired to prevent mutation on genes or to inactivate the mutagenic agent. It seems that the interest in antimutagenic substances displaying multiple mechanisms of action will be an important trend in the research and development of new antimutagenic compounds in the near future. Therefore, this chapter displays various possible mechanisms of action for antimutagenic agent and introduces different types of antimutagens, natural and synthetic, that are considered very important.

Keywords: mutagenesis, antimutagenic, mechanism, natural, synthetic, DNA

1. Introduction

Mutagenicity is the process of induction of permanent heritable changes in the DNA sequence of living systems [1]. It is caused mainly by the external factors, including chemical and physical agents, or can also occur spontaneously due to errors in DNA repair, replicationand recombination [2]. A number of mutagens have been recognized in our environment recently as many factors which modulate the toxic activities either in vitro or in vivo [3]. Agents contributing to mutagenesis in the environment could be from wide-spectrum applications of biocides in the agriculture, industrial sources, and other contaminants [3].

These mutagenic chemicals have severe drawbacks in humans such as cancer and various inherited diseases; therefore, it is important to detect such mutagenic agents precisely and rapidly and also look for solutions to combat them [2].

Natural occurring dietary antimutagens such as healthy protective foods such as fruits and vegetables could strongly counteract the deleterious effect of these mutagens [4]. Additionally, the World Health Organization (WHO) revealed that one-third of all cancer death incidences are preventable depending on the diet type especially health protective phytochemicals that provide an effective solution to these concerns [4]. The current chapter will present the mutagenic events and a brief compilation of the existing scientific findings either from dietary sources or synthetic agents that have the potential activity to combat the disorders

caused by the mutagenic agents, putting in mind possible future perspectives and mechanism of antimutagenics [2].

2. Mechanisms of action

Several classes of antimutagenic compounds may be distinguished based on their mechanism of action as the following:

2.1 Antimutagens with antioxidant potency

Reactive oxygen species (ROS) are generated by many mutagens; therefore, the removal of reactive molecules is considered an important strategy in the process of antimutagenesis. It is reported that compounds with antioxidant propertiescan remove ROS before these molecules react with DNA, resulting in a mutation [5].

It was reported that the antigenotoxic effects of Lipoic acid (LA) (**Figure 1**) against mitomycin-C induced chromosomal aberrations, sister chromatid exchanges, and micronucleus formation was observed in human peripheral lymphocytes. Moreover, LA exhibits both anticlastogenic and antimutagenic activity [6].

2.2 Interaction with mutagen

A potential protective mechanism against mutagenesis is related to the direct chemical interaction between a mutagen and an antimutagenic compound before it induces DNA damage leading to the inhibition of their damaging activity. Sulfhydryl compounds, such as cysteine, can inactivate 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) (**Figure 2**) [7].

2.3 Antimutagen as blocking agents

The mechanism of action for this type of antimutagenics is to prevent mutagenic compounds from reaching target sites such as nucleophilic bichalcophenes (**Figure 3**). They might be able to bind to DNA and, therefore, protect genetic materials from electrophilic mutagenic agents [8].

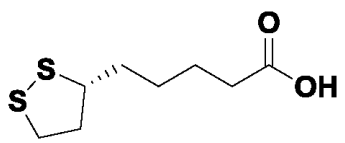

Figure 1.
Lipolic acid.

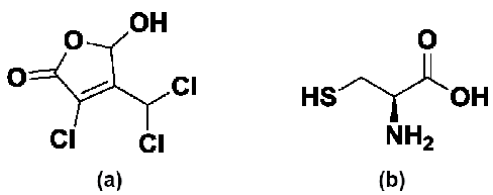

Figure 2.
(a) Mutagen (MX) and (b) antimutagen (cysteine).

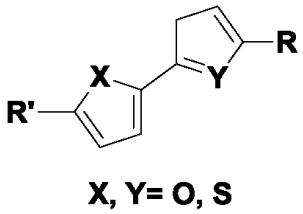

Figure 3.
Bichalcophene derivatives.

2.4 Multifunctionally acting antimutagens

Various antimutagenic agents work through multiple mechanisms affording protection against several mutagens. Noteworthy, the ability of compounds to affect mutagens simultaneously in varied ways significantly enhances antimutagenic effectiveness. Hence, searching for such multifunctionally acting antimutagens is of great importance [9].

2.5 Desmutagenesis

This way of preventing induced cellular mutagenesis depends on mutagens that are inactivated before they can attack the DNA in vitro [3].

2.6 Bio-antimutagenesis

Damaged DNAusually requires fixation steps (e.g., DNAreplication and/or repair) before it can be expressed as stable and heritable mutant genes. Hence this mechanism relates to interference with some aspects of cellular DNA fixation processes working on reducing genetic damage in DNA [3].

3. Antimutagenic agents

Antimutagenic agents are able to combat the disorders caused by mutagens [10]. This group of agents includes both natural and synthetic compounds categories [1].

3.1 Natural antimutagenic agents

The antimutagenic effect of natural sources was investigated due to certain compounds in them or due to whole extract.

3.1.1 Isolated compounds

3.1.1.1 Cinnamaldehyde

It is the first naturally occurring organic antimutagen [11]; it has been involved in screening and chemical studies of such biologically active substances [12]. Antimutagenic action is attributed to either by a selective killing effect of cells which have premutation lesion of DNA via inhibition of the errorprone SOS repair system, or by enhancement of the error-free DNA repair system (**Figure 4**) [13].

3.1.1.2 Punicalagin (PC) and ellagic acid (EA)

Punicalagin is an ellagitannin found in the fruit peel of *Punica granatum*. PC and EA (**Figure 5a, b**) had antioxidant and antigenotoxic properties which dose-dependently and markedly antagonized the effect of tested mutagens such as NaN_3, benzo[a]pyrene, 2-aminoflourine, and methyl methanesulfonate (EMS), with 90% mutagenicity inhibition [14].

3.1.1.3 Luteolin derivatives

Luteolin derivatives (luteolin 7-O-rutinoside, luteolin 7-O-glucoside, and luteolin 7-O-glucuronide) (**Figure 6**) are isolated from *Mentha longifolia* (L.) to evaluate the antimutagenic activities by using Ames *Salmonella* test (TA 1535 and TA1537 strains). The antimutagenic activity on TA1537 was 87.63, 84.03, and 67.77%, respectively. The antimutagenic activity of these compoundscan be due to

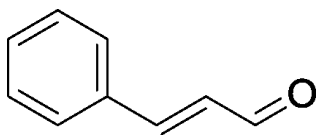

Figure 4.
Cinnamaldehyde.

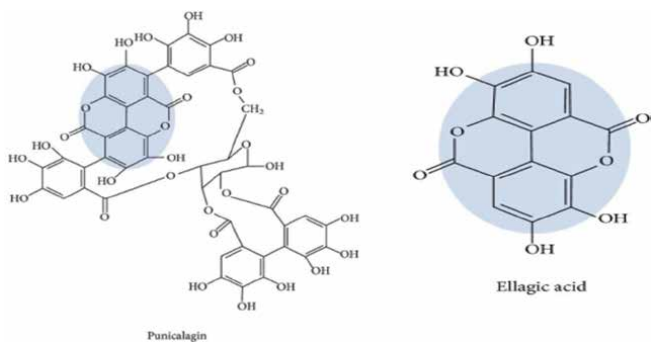

Figure 5.
(a) Punicalagin (b) ellagic acid.

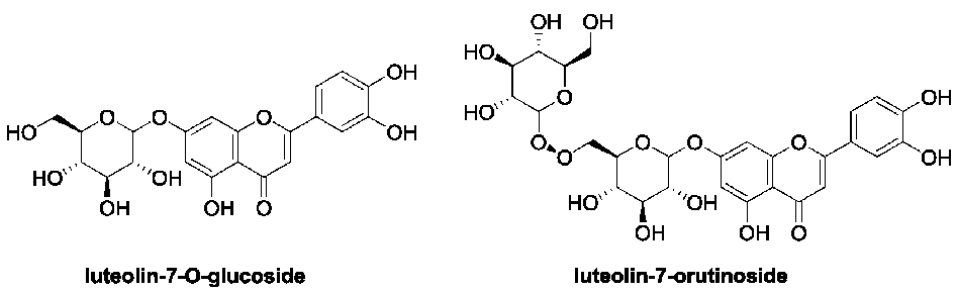

Figure 6.
Luteolin derivatives.

the inhibition capability by blocking 9-aminoacridine binding to DNA [15]. In addition, the inhibition effects against ethyl methanesulfonate may be related to the protection against DNA double-strand breaks or EMS alkylating action (**Figure 6**) [16].

3.1.1.4 Acetogenins

Annona crassiflora Mart. (AcM) is a Brazilian plant, *araticum*, which is widely used as a therapeutic medicine to treat several diseases such as rheumatism, diarrhea, and syphilis. Ethanolic extract were evaluated for antimutagenic and cytotoxic effects. The results indicated an antimutagenic activity of the AcM due to the presence of acetogenins (**Figure 7**) and other flavonoids [17].

3.1.1.5 Pinocembrin and cardamonin

Pinocembrin and cardamonin (**Figure 8**) are found in Sozuku (Chinese drug from dried seed of *Alpinia katsumadae* HAYATA). These compounds showed potent antimutagenic activity against 2-amino3,4-dimethylimidazo-[4,5-f] quinolone (MeIQ) mutagenesis in Ames test using the *S. typhimurium* TA100 and TA98 strains [18].

3.1.1.6 Harpagoside (HS)

It isa type of iridoid glycoside. HS (**Figure 9**) is considered as the main active component extracted from *Harpagophytumprocumbens* (HP) which is used as anti-inflammatory and analgesic particularly against painful osteoarthritis. The extract wastested to evaluate the antimutagenic activity of HS and HP against mutagenic activity of 1-nitropyrene (1-NPy) that is one of the most abundant nitro-polycyclic aromatic hydrocarbons particularly in diesel exhausts. The results showed that HS significantly reduced the mutagenicity of 1-NPy in pretreatment and particularly in co-treatment. Moreover, HP extract significantly reduced the genotoxicity [19].

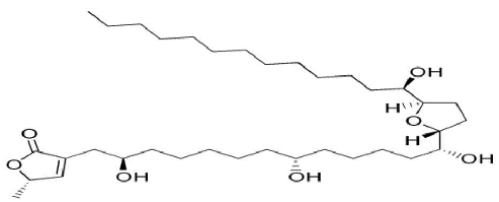

Figure 7.
Acetogenins.

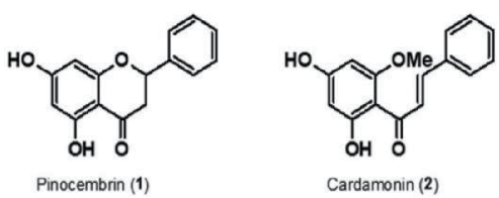

Figure 8.
Pinocembrin and cardamonin.

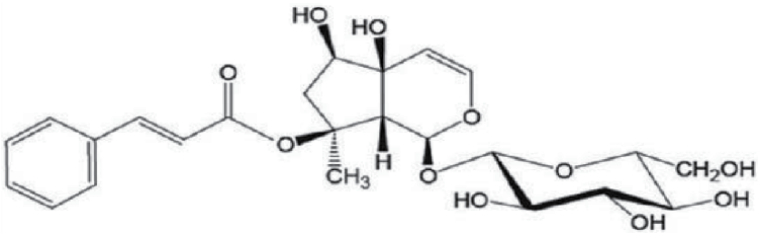

Figure 9.
Harpagoside (HS)

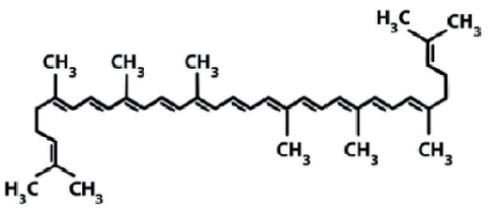

Figure 10.
Lycopene.

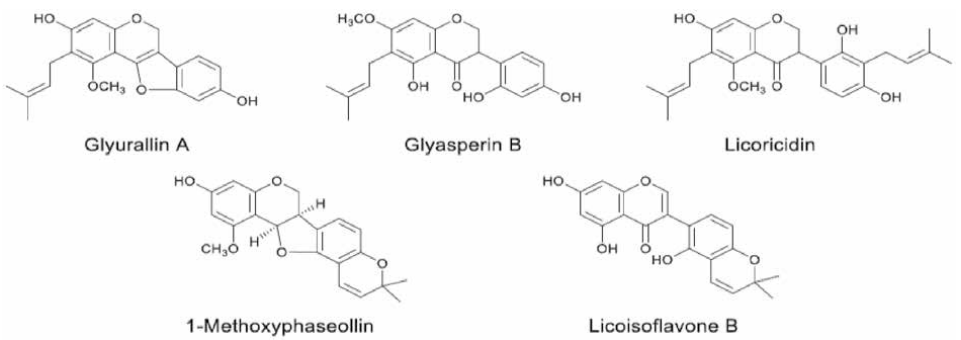

Figure 11.
Glycyrrhiza aspera root extract.

3.1.1.7 Lycopene

Natural oleoresin is rich in lycopene (**Figure 10**), which was obtained from two types of tomato (Zedona and Gironda). The antimutagenic activity of oleoresin was tested against aflatoxin B1 (AFB1), and both varieties had awfully high antimutagenic potential against AFB1 (60–66%) [20].

3.1.1.8 Compounds extracted from Glycyrrhiza aspera *root*

The powdered extract of *G. aspera* root was assayed for antimutagenic activity against N-methyl-N-nitrosourea (MNU) in S. typhimurium TA1535. Five components that were extracted by using ethanol which had antimutagenic activity against MNU were identified as glyurallin A, glyasperin B, licoricidin, 1-methoxyphaseollin, and licoisoflavone B (**Figure 11**). These components were demonstrated to possess an antigenotoxic effect against carcinogenic MNU. So this extract can be used to prevent DNA damage by N-nitrosamines for cancer chemoprevention [21].

3.1.2 Plant extract

3.1.2.1 Date palm *fruit aqueous extract*

It was found that *date palm* extract displays strong antimutagenic activity against ultraviolet (UV) radiation, and mitomycin C-induced mutagenesis, when it was analyzed using *E. coli* RNA polymerase β-based rifampicin resistance assay, but did not show any significant antimutagenesis against ethyl methane sulfonate (EMS) [22].

3.1.2.2 Maytenus ilicifolia *and* Peltastes peltatus *extract*

These two plants are both rich in compounds of the tanninand flavonoid groups and frequently employed in folk medicine. Antimutagenicity was determined against known mutagenic substances such as 4-oxide-1-nitroquinoline, NaN_3, aflatoxin B1, 2-aminofluorene and 2-aminoanthracene, and 2-nitrofluorene using the *Salmonella*/microsome assay. There was a significant decrease in mutagenicity for the tested extract by 75%.The mechanism of antimutagenicity of this extract is still under study [23].

3.1.2.3 Citrus limonum *fruit residues (CLFR)*

Aqueous and acidified methanol extracts of CLFR were evaluated for their total phenolic contents and antioxidant and antimutagenic activities. Antimutagenic potential of the extracts was done by Ames test. The results supported that the extracts from CLFR were mutagenically safe due to its high phenolic content which can act as antioxidant and anitmutagenic [24].

3.1.2.4 Mimosa tenuiflora *(MT) extract*

The genotoxic effect of MT was investigated by using both micronucleus test and Ames test in *Salmonella typhimurium* TA97, TA98, TA100, and TA102, respectively. The results showed that the extract did not induce mutations in any strain. Further studies of toxicity were performed to investigate the use of this plant in the treatment of diseases [25].

3.1.2.5 Albeofructus *(ADA) extract*

It is an extract of *Acanthopanax divaricatus* which possesses antimutagenic activity against direct-acting mutagenic agents through the rapid elimination of mutagenic compounds from the cells before the induction of genetic material damage [26].

3.1.2.6 Anemopsis californica *(AC)*

Although *A. californica* (AC) possesses therapeutic uses, so it could be useful for reducing genotoxic risk generating from ROS-agents exposure and provide protection against poly-cyclic aromatic hydrocarbons which are well known as premutagens and precarcinogens [27].

3.1.2.7 Citrus sinensis *and* Citrus latifolia

The essential oils of *Citrus sinensis* and *Citrus latifolia* showed antimycotic besides antimutagenic and antioxidant activity. Their main components are R-(+)-limonene, α-myrcene, β-thujene, and γ-terpinene [28].

3.1.2.8 Heterotheca inuloides *(HI) extract*

The methanolic extract of HI reduced the mutagenicity of benzo[a]pyrene, norfloxacin, and 2-aminoanthracene. The antigenotoxic properties could be due to the antioxidant properties of component into extract such as catenanes, sterols, polyacetylenes, triterpenes, sesquiterpenes, flavonoids, and flavonoid glycosides [29].

3.1.2.9 Extracts of Acacia salicina

Literatures revealed that this extract displayed potent antioxidant and antimutagenic activities [30]. Also chloroform extract showed antimutagenic effect against both direct- and indirect-acting mutagens, as the extract may act as a blocking agent that is capable of influencing the activities of enzymes engaged in the metabolism of mutagens and carcinogens. Moreover, the tested extract displayed the ability to react directly with the mutagens electrophilic metabolite sand was capable of protecting against oxidative DNA damage [30].

3.1.2.10 Wheat bran

It was reported that wheat bran provides antimutagenic effects that related to the presence of the antioxidant phytic acid. It was demonstrated that phytic acid may intercept carcinogenic azoxymethane, inhibiting it even before it can damage DNA. Moreover, antioxidants included in wheat bran are able tomodulate DNA repair enzymes [31].

3.1.2.11 Vegetables

Activity was displayed by beets, chives, horseradish, onions, rhubarb, and spinach. All cruciferous vegetables showed strong to moderate antimutagenic activities, except Chinese cabbage, which displayed weak activity. Moderate antimutagenicity was found in green beans and tomatoes, whereas weak activities in egg plant, garden cress, many types of lettuces, leeks, mangold, cucumber, pumpkin, radish, and summer squash. However, some vegetables such as *Asparagus*, carrots, fennel leaves, parsley, green pepper, and radishes were not found to display any antimutagenicity [32].

Antimutagenic activity of many vegetable juiceswere earlier studied againstmutagenicity induced by2-amino-3-methyl[4,5-f]-quinoline (IQ), 2-amino-3,4dimethylimidazo[4,5-f] quinoline (MeIQ) or 2-amino3,8-dimethylimidazo [4,5-f] quinoxaline (MeIQx) in S.typhimurium TA98 and TA100 [33].

3.1.2.12 Fruits

Current research all over the world has focused on health protectiveproperties of fruits including antimutagenic potential of different fruittypes and their cultivars. Concerning apple fruit, its antioxidant and radioprotective properties were found to be better correlated with its antimutagenic effect [34]. Recently, copaiba, an exotic

Brazilian fruit, possesses the antimutagenic potential of copaiba powder (dose of 100 mg/kg) showing great reduction of micronuclei [35].

3.1.2.13 Other sources

3.1.2.13.1 Ganoderma lucidum

Ganoderma lucidum was extracted with hot water (GLW) and then partially purified with crude glycoside extract (GLG) and crude polysaccharide extract (GLP). The extract was tested to evaluate the antioxidant and antimutagenic activity. The results showed that the extract has antimutagenic activity due to β-glucan content and antioxidant action due to the presence of high polyphenolic content [36].

3.1.2.13.2 Macro fungus

It was demonstrated that ethyl acetate extract of macro fungus showed the in vitro antimutagenic activity of *Phellinus rimosus*. The activity of the extract against direct-acting mutagens may result from the direct inactivation of mutagens. It is probable that, due to stimulation of the transmembrane export system in bacteria, mutagenic compounds are removed from the cells before they influence the DNA structure [37]. Additionally, in the case of doxorubicin (DXN), the extract of *P. rimosus* may affect the intercalation of mutagens to genetic material.

3.2 Synthetic antimutagenic agents

Synthetic antimutagens is another important trend in the area of antimutagenicity research.

3.2.1 Steroidal hormonal molecules

Bile acids have either a co- or an antimutagenic activity toward various direct- and indirect-acting mutagens [38]. It was reported that steroidal hormones could inhibit the genotoxicity of both direct- and indirect-acting mutagens [39]. For example, both ethinyl oestradiol and mestranol (**Figure 12**), which are synthetic derivatives of 3-estradiol largely used in contraceptive pills, are strong mutagenic inhibitors acting at nanomolar concentrations [39].

3.2.2 Gallic acid

It could act as a nucleophile to scavenge the electrophilic mutagens. It was implied that gallic acid (**Figure 13**) can bind or insert into the outer membrane

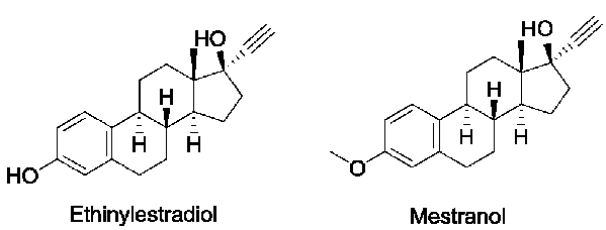

Figure 12.
Steroidal hormonal molecules

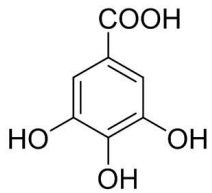

Figure 13.
Gallic acid.

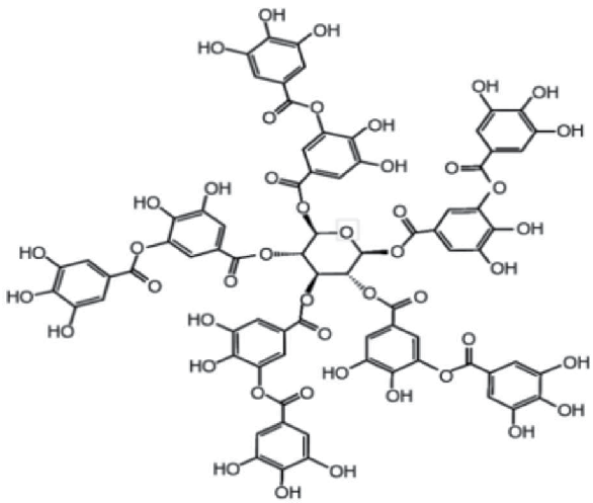

Figure 14.
Tannic acid.

transporters leading to the blockage of a mutagen that was transferred intothe cytosol [40]. One of the mutagenic substances that gallic acid affects is NaN3. It is widely used in agriculture, industry, and medicine, but it is a highly toxicsubstance. If sodium azide is found in the intracellular milieu, azide ions bind Fe3þ in hemoglobin and inhibit the respiratory chain of metabolism [41].

3.2.3 Tannic acid

The anticlastogenic effect of tannic acid (**Figure 14**) was studied *in vivo* in the mouse micronucleus test. Moreover, the antimutagenic effect of tannic acid was investigated *in vivo* in the mouse spot test using male PW and female C57BL/10 mice. The results showed that tannic acid can act as an anticlastogen and antimutagen in vivo [42].

3.2.4 Synthesized β- aminoketones

Theantigenotoxic potential of two newly synthesized β-aminoketones such as2-{(4-bromophenyl)[(4-methylphenyl) amino] methyl} cyclohexanone and 2-{(4-chlorophenyl)[(4-methylphenyl) amino] methyl} cyclohexanone compounds was tested against the mutagenN-methyl-N-nitro-N-nitrosoguanidine (MNNG), acting by DNAmethylation (**Figure 15**) [9]. The antimutagenic potential of these compounds may be related to the inhibition of the production of O6-methylguanine,

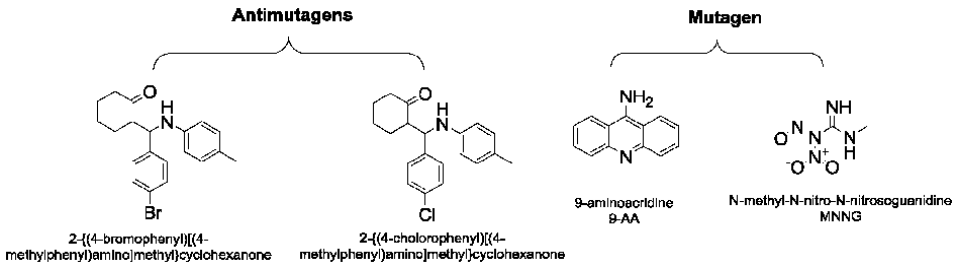

Figure 15.
β-Aminoketones and mutagens.

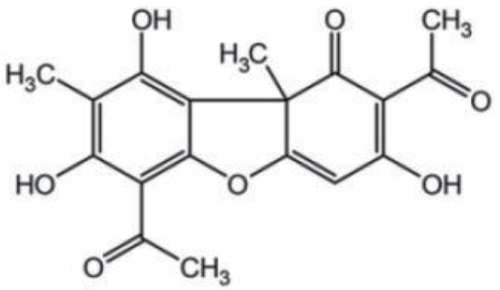

Figure 16.
Usnic acid.

a product of MNNG that is related to its mutagenic effect. Both compounds also abolished mutagenesis induced by 9-AA that binds to DNA noncovalently by intercalation [43].

3.2.5 Phenolic agents

This category of antimutagenics acts against mutagens via either intracellularor extracellular mechanisms [44]. The extracellular mechanism showed interference with the cytochrome P450-mediated metabolismof these mutagens and the interaction with active mutagenicmetabolites [8]. Moreover, the antimutagenic potency of these compounds may be relatedto DNA protection from mutagens presenting electrophilicproperties [8].

Hydroxyphenyliminoligands and their metal complexes [Cu(II), Co(II), Ni(II) and Mn(II) complexes] of usnic acid (**Figure 16**) which is isolated from *Usnea longissima*, were synthesized by Schiff base method with *O-, P-,* and *M-* aminophenol compounds to determine their antimutagenic activity against different bacteria species. The results showed that the Co and Mn complexes of the ligands possess potent antimutagenic activity [45].

New polymeric microspheres containing azomethine were designed and synthesized to evaluate their antimutagenic activity against NaN_3, among of them; a new polymeric microspheres containing azomethine (**Figure 17**) which contains R = CH_3 had potent antimutagenic effect against NaN_3 [46].

Chitosan derivatives containing quaternary ammonium groups and di (tertbutyl) phenol (TBPh) (**Figure 18**) in the polymer side chain improved the antimutagenic efficiency of the polymer from 48 to 93% [47].

Hydrazone derivatives were synthesized to study their antioxidant and antimutagenic activity against 4-NPD and NaN3 in *S. typhimurium* TA98 and TA100, respectively, among of them; the hydrazone derivative (**Figure 19**)

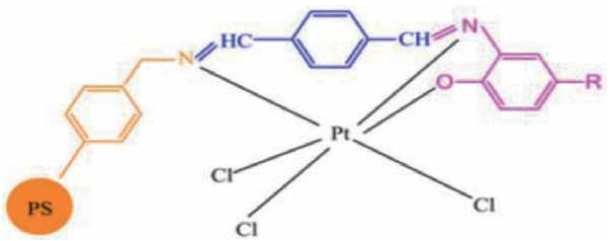

Figure 17.
New polymeric microspheres containing azomethine.

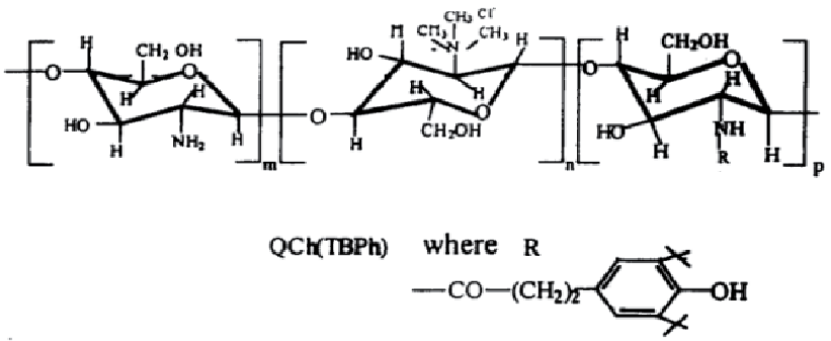

Figure 18.
Chitosan derivatives containing quaternary ammonium groups.

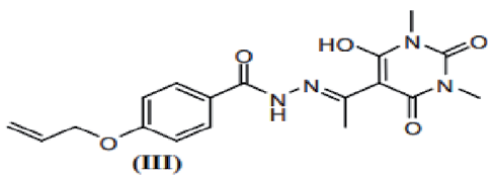

Figure 19.
Hydrazone derivatives

had high antimutagenic activity. The strongest antimutagenic activity was observed at 5 mg/plate concentration against *S. typhimurium* TA100 strain [48].

3.2.6 Xanthones

The potential antimutagenic of xanthonesis attributed to different mechanisms, such as the rapid elimination of mutagens from bacteria; the interaction between antimutagens and the reactive intermediates of mutagens; and the influence on microsomal enzymes against direct mutagen 4-nitroquinoline-N-oxide (NQNO) (**Figure 20**) [49].

3.2.7 Indols

Novel polymeric-Schiff bases including indol (L1, L2, L3) (**Figure 21**) exhibited the antigenotoxic properties against sodium azide in human lymphocyte cells by micronuclei (MN) and sister chromatid exchange tests [50].

A series of indolizine derivatives have been synthesized to determine their anti-mutagenic activity, the indolizine derivative (**Figure 22**) had the highest activity [51].

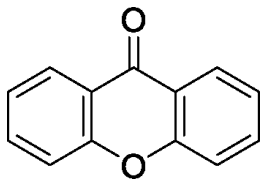

Figure 20.
Xanthone.

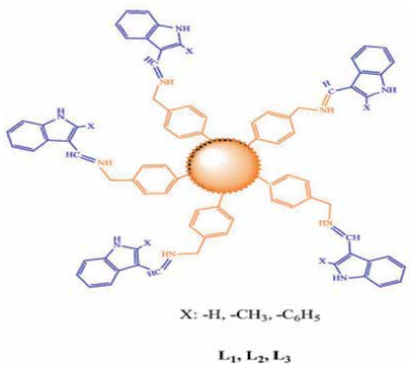

Figure 21.
Novel polymeric-Schiff bases.

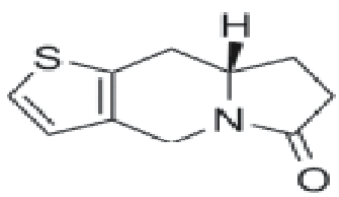

Figure 22.
Indolizine.

3.2.8 Organoselenium

Scientists demonstrated that this series of compounds are protected against genotoxicity and oxidative stress induced by an indirect-acting mutagen CP [52]. This is attributed to effect of CP on DNA through its alkylating properties and free radicals production [53].

3.2.9 Bichalcophenes

The novel bichalcophenes significantly decreased the mutagenicity induced by two mutagens, namely, NaN_3 and BP [54]. It was found that the antimutagenic potential of the compounds could be attributed to their antioxidant activity [55].

3.2.10 Others

New zerumbone-bicarbonyl analogues were synthesized to determine their antimutagenic activity against *Salmonella* tester strains. Zerumbal (**Figure 23**) had significant higher antimutagenic activity than zerumbone [56].

Two newly synthesized oxadiazoles: 1,3-bis(5-benzylthio-1,3,4-oxadiazol-2-yl) benzene (M1) and 1,4-bis(5-benzylthio-1,3,4-oxadiazol-2-yl) benzene (M2) (**Figure 25**) were synthesized and studied in *Salmonella typhimurium* strains TA97, TA100, TA102 and TA1537 in the presence and absence of S9mix. The antimutagenicity of M1 and M2 against H_2O_2, NaN_3, and 4-nitro-o-phenylene diamine (NPD) using the tester strains, was also investigated. The two compounds were found to be nonmutagenic [58].

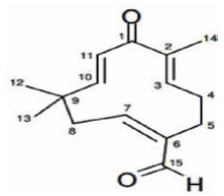

Figure 23.
*1,4-Dihydropyridines (1,4-DHP) (**Figure 24**) possessed antioxidant and antimutagenic activities. The compounds modified the activity of DNA repair enzymes, to protect the DNA in living cells against peroxynitrite-induced damage [57].*

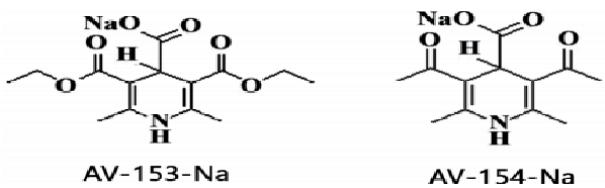

Figure 24.
1,4-Dihydropyridines (1,4-DHP) derivatives.

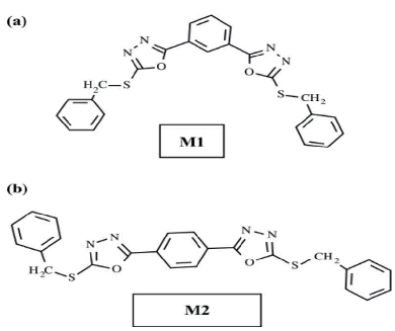

Figure 25.
Oxadiazole derivatives.

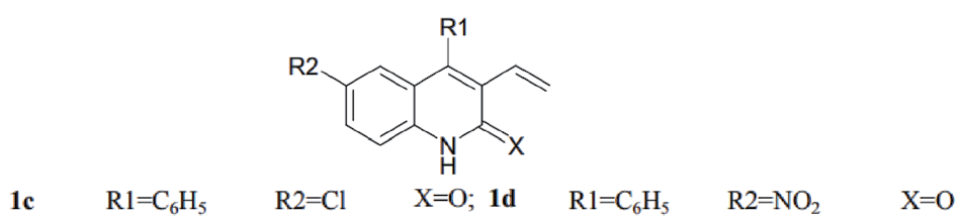

Figure 26.
Dihydrothienoquinoline derivatives.

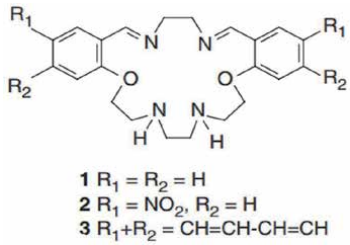

Figure 27.
Azacrown ether Schiff bases.

Dihydrothienoquinoline derivatives were designed and synthesized to evaluate their antimutagenicity using Ames test. Several compounds showed good antimutagenicity. The results for compounds (**Figure 26**) were found to be statistically significant (P = 0) [58].

A series of novel azacrown ether Schiff bases have been synthesized, and they were investigated for their antimutagenic activities using the spot test and Ames test using strains TA1535, TA100, and TA97a of *Salmonella typhimurium*. The results showed that compounds 1 and 2 (**Figure 27**) were antimutagenic [59].

Conflict of interest

The authors declare no conflict of interest.

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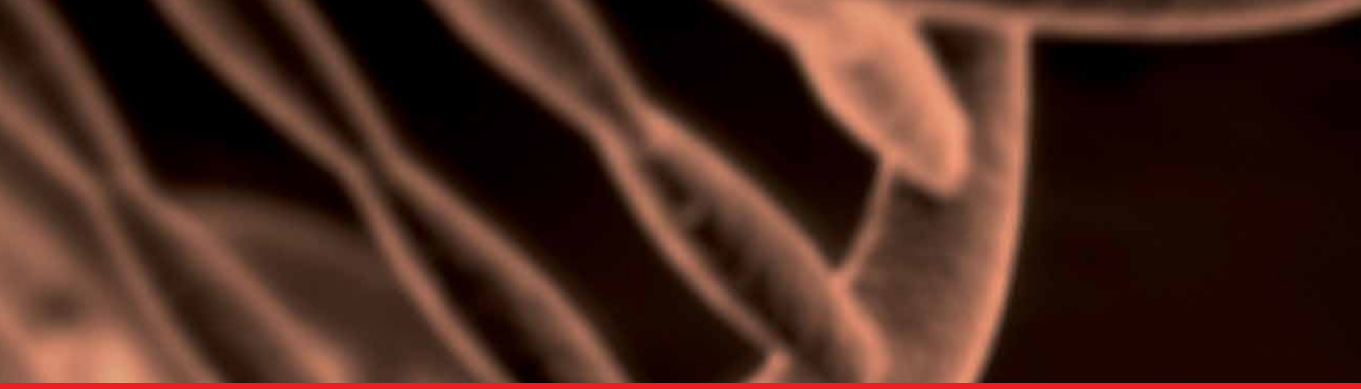

Edited by Sonia Soloneski and Marcelo L. Larramendy

This edited book, "*Genotoxicity and Mutagenicity - Mechanisms and Test Methods*", aims to present the latest developments from different fields, highlighting the detrimental influence that mutagenic and genotoxic agents inflict on DNA and how antimutagenic and anticarcinogenic modulators are able to reduce the negative impact of such toxic agents on living species.

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