Epigenetic side-effects of common pharmaceuticals: A potential new field in medicine and pharmacology

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SUMMARY

The term "Epigenetics" refers to DNA and chromatin modifications that persist from one cell division to the next, despite a lack of change in the underlying DNA sequence. The "epigenome" refers to the overall epigenetic state of a cell, and serves as an interface between the environment and the genome. The epigenome is dynamic and responsive to environmental signals not only during development, but also throughout life; and it is becoming increasingly apparent that chemicals can cause changes in gene expression that persist long after exposure has ceased. Here we present the hypothesis that commonly-used pharmaceutical drugs can cause such persistent epigenetic changes. Drugs may alter epigenetic homeostasis by direct or indirect mechanisms. Direct effects may be caused by drugs which affect chromatin architecture or DNA methylation. For example the antihypertensive hydralazine inhibits DNA methylation. An example of an indirectly acting drug is isotretinoin, which has transcription factor activity. A two-tier mechanism is postulated for indirect effects in which acute exposure to a drug influences signaling pathways that may lead to an alteration of transcription factor activity at gene promoters. This stimulation results in the altered expression of receptors, signaling molecules, and other proteins necessary to alter genetic regulatory circuits. With more chronic exposure, cells adapt by an unknown hypothetical process that results in more permanent modifications to DNA methylation and chromatin structure, leading to enduring alteration of a given epigenetic network. Therefore, any epigenetic side-effect caused by a drug may persist after the drug is discontinued. It is further proposed that some iatrogenic diseases such as tardive dyskinesia and drug-induced SLE are epigenetic in nature. If this hypothesis is correct the consequences for modern medicine are profound, since it would imply that our current understanding of pharmacology is an oversimplification. We propose that epigenetic side-effects of pharmaceuticals may be involved in the etiology of heart disease, cancer, neurological and cognitive disorders, obesity, diabetes, infertility, and sexual dysfunction. It is suggested that a systems biology approach employing microarray analyses of gene expression and methylation patterns can lead to a better understanding of long-term side-effects of drugs, and that in the future, epigenetic assays should be incorporated into the safety assessment of all pharmaceutical drugs. This new approach to pharmacology has been termed "phamacoepigenomics", the impact of which may be equal to or greater than that of pharmacogenetics. We provide here an overview of this potentially major new field in pharmacology and medicine.

Introduction

Definition of epigenetics

The word "epigenetics" has had many definitions, and its meaning has changed over time. Initially it was used in a broad sense, but has become more narrowly linked to specific molecular phenomena occurring in organisms [1]. Epigenetics, as in "epigenetic landscape", was first coined by Waddington in 1942 as a portmanteau of the words "genetics" and "epigenesis" [2]. "Epigenesis" is an older word used to describe the differentiation of cells from their initial totipotent state in embryonic development. When Waddington coined the term, the physical nature of genes and their role in heredity was not yet known, so he used it as a conceptual model of how genes might interact with their surroundings to produce a phenotype. Holliday subsequently defined epigenetics as "the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms" [3].

The modern usage of the word is narrower, referring to heritable traits in cells and organisms that do not involve changes to the underlying DNA sequence [4]. The Greek language prefix "Epi" denotes features that are "above" or "in addition to" something; thus epigenetic traits exist on top of, or in addition to, the traditional

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molecular basis for inheritance. Hence the modern meaning of "epigenetics" basically refers to changes in gene expression in cells and organisms. These changes may persist through cell division and for the remainder of the cell's or organism's life. Sometimes the changes last for multiple generations of the organism, and are known as "transgenerational" effects [5], but again, there is no change in the underlying DNA sequence. Rather, environmental factors cause the organism's genes to behave, or "express themselves", differently [6]. A good example of epigenetic change is the process of cellular differentiation. During morphogenesis, totipotent stem cells become the various pluripotent cell lines of the embryo, which in turn become fully differentiated cells [7,8]. In other words, a single fertilized egg cell, the zygote, changes morphology over multiple divisions into the many cell types of the organism, such as neurons, liver cells, epithelium, blood vessels, etc., as it continues to divide. It does so by a process of activating some genes, while silencing others [7,8]. Regenerating totipotency during development of germ cells or nuclear transfer (cloning) entails re-expression of pluripotency-specific genes and extensive erasure of epigenetic modifications [8].

The similarity of the word to "genetics" has generated many parallel usages. The "epigenome" is a parallel to the word "genome", and refers to the overall epigenetic state of the genome. The phrase "genetic code" has also been adapted to the "epigenetic code" and has been used to describe the set of epigenetic features that create different phenotypes in different cells. Likewise "genomics" becomes "epigenomics", the study of epigenetic modification at a level much larger than a single gene, including whole-genome epigenetic scanning technologies, and the detection of quantitative alterations, multiplex modifications, and complex regulatory sequences outside of genes [9].

Relevance of epigenetics to modern medicine

In the past few years, several pioneering studies have brought epigenetics to the forefront of molecular biology, and this rapidly growing field has been the subject of several excellent reviews [6,10–13]. Interest has dramatically increased as it has become clear that epigenetics will be essential to understanding many topical biological phenomena such as stem cells [14], nuclear transfer (cloning) [15], cellular reprogramming [16], aging [17], evolution and speciation [18], and agriculture [19].

Also, it is becoming clear that a wide variety of common illnesses, behaviors, and other health conditions may have at least a partial epigenetic etiology, including cancer, respiratory, cardiovascular, reproductive, and autoimmune diseases [10], neurological disorders such as Parkinson's, Alzheimer's, and other cognitive dysfunctions [10,20], psychiatric illnesses [21], obesity and diabetes [22], infertility [23] and sexual dysfunction [24]. Effectors of epigenetic changes include many agents, such as heavy metals, pesticides, tobacco smoke, polycyclic aromatic hydrocarbons, hormones, radioactivity, viruses, bacteria [10], basic nutrients [25], and the social environment [26], including maternal care [27]. It has even been suggested that our thoughts and emotions can induce epigenetic changes [28,29].

Disease nosology as a "fuzzy" concept within an epigenetic framework

Exactly how the environment changes gene expression at the molecular level and how this can lead to disease are being explored in novel approaches to environmental health research [6,30]. Indeed, the very definitions of health and disease may be redefined, or at least blurred, when considered in an epigenetic context, since the boundary between the two states is obviously not a strict one, but rather "fuzzy" when considered from the perspective of gene expression [31]. Epigenomic studies will thus further the develop-

ment of theories concerned with the application of non-Aristotelian concepts (that violate principles of classical logic) to the diagnosis and nosology of illness [32,33]. And high-throughput epigenetic screens will surely have an enormous impact on understanding the causes of "complex" non-Mendelian disease [34–37]. For example many "Functional Somatic Syndromes" such as chronic fatigue syndrome, post-traumatic stress disorder, fibromyalgia, 'sick building syndrome', chronic Lyme disease, etc. that are currently hard to diagnose, medically explain, and treat because they are outside the traditional model of disease as a single entity, might only be understood at the mechanistic level by applying concepts of systems biology and epigenetics [38,39].

A diverse variety of epigenetic processes have been identified

Many types of epigenetic processes have been identified, such as methylation of DNA [10], and acetylation, phosphorylation, ubiquitylation, and sumolyation of histones [10]. Other types of regulation that might be termed epigenetic, since they provide an extra layer of transcriptional control are: gene translocation, DNA repair, RNA transcription, RNA stability, alternative RNA splicing, protein degradation, gene copy number, and transposon activation [30]. Recently the effects of miRNAs on epigenetic machinery, and the control of miRNA expression by epigenetic mechanisms have been explored [40]. Other epigenetic mechanisms are likely to be discovered as research advances.

In summary: epigenetic processes are natural and essential to the function of organisms, but if they occur improperly, there can be major adverse health and behavioral effects.

The hypothesis

Although it is now becoming well-established that various environmental agents can cause epigenetic changes, one class of compounds that has been largely absent from most studies so far, is pharmaceutical drugs. Based on our rapidly-accumulating knowledge of gene/environment interactions, it stands to reason that drugs in current therapeutic practice would affect the epigenomic state of genes.

Therefore, the fundamental hypothesis of this article is that commonly-used pharmaceutical drugs can cause persistent epigenetic changes, which can be manifested in the persistence of drug-induced adverse events (side-effects). Below we evaluate the evidence for this hypothesis, and discuss the consequences for modern medicine should it prove to be correct.

Evaluation of the hypothesis

Here we provide examples of drugs for which there is clinical and/or experimental evidence for epigenetic effects as a direct effect on DNA methylation or histone acetylation, or indirect effect on transcription factor activation or receptor expression, etc. We also consider drugs which have been documented to cause persistent side-effects, but for which an epigenetic etiology for such effects has yet to be proven. A full list of drugs described here, with their attendant known or postulated epigenetic effects and/or side-effects is shown in Table 1.

Direct effects

Hydralazine, procainamide, valproate, and methotrexate

Some drugs which had an unknown mechanism of action when they were first introduced are now known, remarkably, to effect DNA methylation or histone acetylation. Two examples of the former are the drugs hydralazine, a vasodilator used to treat

Table 1
Examples of drugs for which there is clinical and/or experimental evidence for epigenetic effects as a direct effect on DNA methylation or histone acetylation, or indirect effect on transcription factor activation or receptor expression, etc. We also list drugs which have been documented to cause persistent side-effects, but for which an epigenetic etiology for such effects has yet to be proven.

Pharmaceutical drug	Known or postulated epigenetic effects and/or clinical consequences.
Hydralazine & procainamide	Inhibit DNA methyltransferase, drug-induced SLE, autoimmunity
Methotrexate	Alters methionine synthesis, DNA methylation, drug resistance
Valproic acid	Inhibits Histone deacetylase, altered gene expression, skeletal malformation
Thalidomide	Teratogenic, major malformations, sometimes transgenerational
Isotretinoin	Teratogenic, major malformations, hyperlipidemia, ocular problems, alopecia, psychiatric disturbances
Neuroleptics	Tardive dyskinesia, hyperglycemia, diabetes, cognitive dysfunction
Methylphenidate	Altered gene expression, synaptic plasticity, behavioral changes
SSRIs and antidepressants	Altered chromatin architecture, gene expression, behavior, infertility
Chemotherapeutics	Genotoxic, secondary cancers, "chemo brain"
General anesthetics	Cognitive dysfunction, Alzheimer's & Parkinson's disease
Contraceptives	Cancer, osteoprosis, weight gain, sexual dysfunction
Chloroquine antibiotics	Psychiatric illnesses, cardiac arrythmias, dysglycemia, tendon ruptures
Beta-blockers	Hyperglycemia, diabetes, insomnia, depression
Statins	Myalgia, muscle cramps, muscle and liver damage, gastrointestinal issues
Cox-2 inhibitors	Persistently increased cardiovascular mortality risk

hypertension, and procainamide, an antiarrythmic sodium channel blocker, both of which inhibit DNA methylation and can trigger a lupus-like autoimmune disease [41]. The lupus-like disease and autoimmunity is thought to result from the body mounting an immune response against its own tissues because of inappropriate expression of proteins caused by extensive genomic hypomethylation [42,43]. Interestingly, antibodies are frequently found against chromatin, and such anti-DNA antibodies are used in diagnosis [44]. It was later shown that procainamide inhibited DNA methylation by inhibiting the DNA methyltransferase reaction [45], specifically DNA methyltransferase I [46], while hydralazine may interfere with DNA methyltransferase activity directly, or influence expression of the DNA methyltransferase gene [47].

Another example of a known drug commonly used in therapy that was not initially known to cause epigenomic effects is valproate (valproic acid, Depakote). Valproate is a known antiepileptic and mood-stabilizing drug that was believed to act on GABAergic neurons. However, recent studies revealed the unexpected finding that it is actually a histone deacetylase inhibitor, which can alter chromatin structure by increasing histone acetylation [48]. It has also been shown recently that it targets demethylation activity to ectopically methylated DNA resulting in replication-independent demethylation of DNA [49]. The resultant epigenetic reprogramming is widespread and involves demethylation of specific genes [50]. One of the side-effects of valproate therapy is hepatotoxicity, such as microvesicular steatosis and necrosis of the liver. Gene expression profiling data of mouse livers treated with valproate showed changes in the expression of genes associated with lipid, fatty acid, and steroid metabolism, oncogenesis, signal transduction, and development [51]. Valproate is also a potent teratogen, and valproate-induced skeletal malformations were also associated with large alterations in gene expression cascades [52]. Since valproate action does not require cell division, it potentially affects the epigenomic status of genes in mature post-mitotic cells such as CNS neurons. For example histone deacetylation-dependent transcriptional control is crucial for the regulation of glial cell growth [53]. Data suggests that valproate may induce abnormalities of epigenetic transcriptional regulatory mechanisms in glial cells, resulting in reduced cell proliferation, which may in turn cause cognitive dysfunction or mental illness [53,54].

Drugs might also affect DNA methylation patterns by altering the activity of enzymes involved in the synthesis and metabolism of methyl groups in the cell resulting in a decrease in the concentration of SAM, the methyl donor in the DNA methylation reaction. For example, the enzyme Methylentetrahydrofolate Reductase (MTHFR) regulates folate and methionine metabolism in the cell.

A common polymorphism 677C –>T results in reduced activity of this enzyme and, as a consequence, a reduction in methionine and an increase in homocysteine. Reduced MTHFR activity might induce DNA hypomethylation [55–57] and was proposed to increase the risk for colorectal disease in elder patients or under conditions of low folate intake and high alcohol intake [58]. The anticancer agent methotrexate also affects methionine synthesis. An increased toxicity and increased homocysteine was observed when ovarian cancer patients bearing the 677->T mutation were treated with methotrexate [59]. It stands to reason that DNA methylation was also affected although this was not determined. This is an interesting example of how pharmacogenetics intersects with pharmacoepigenomics.

Epigenomic untoward effects of drugs might also result in drug resistance. Aberrant hypermethylation of gene promoters results in gene silencing [60]. Thus, if a drug causes DNA hypermethylation it might silence the gene encoding its target, resulting in drug resistance. In addition, hypermethylation might result in silencing of critical genes such as tumor suppressors genes. It was shown that a number of drugs used in chemotherapy such as hydroxyurea, topoisomerase II inhibitors etoposide and nalidixic acid, the antibiotic doxorubicin, the microtubule inhibitors vincristine, vinblastine, and colchicines, the DNA cross-linking agent cis-platinum and the antimetabolites 1-beta-Darabinofuranosylcytosine, 5-fluorouracil, 5-fluorodeoxyuridine, and methotrexate were associated with drug-induced DNA hypermethylation in cell culture [61,62]. Anecdotal evidence indicated that this hypermethylation also occurred *in vivo* during high-dose 1-beta-D arabinofuranosylcytosine and hydroxyurea treatments in two leukemic patients [62]. The mechanisms responsible for drug-induced hypermethylation are currently unknown, but appear to be an "indirect" epigenetic effect, as discussed below.

Indirect effects

Teratogens with epigenetic effects: thalidomide and isotretinoin

A wide range of different chemicals are capable of inducing birth defects in humans and in animals, known collectively as "teratogens". It was previously believed that the uterus was impervious and protected the mammalian embryo from all extrinsic factors. However, after the Thalidomide disaster of the 1960s, it became apparent that the developing embryo could be highly vulnerable to some environmental agents. The precise molecular mechanism of most teratogens is unknown, but it is possible that they could interfere with the normal controls of DNA methylation, resulting in aberrant gene expression [63].

The aforementioned Thalidomide is a sedative-hypnotic, and multiple myeloma medication. The drug is a strong teratogen in mammals, including humans. The fetus is susceptible to its effects from the 21st to the 40th day of gestation, and it has the potential to produce malformations of most of the major systems of the body. Approximately 40% of babies with thalidomide embryopathy died of either bowel atresia, heart malformations or renal agenesis [64]. The most common phenotypic effect in thalidomide embryopathy is truncation of the upper limbs. Some thalidomide victims have produced offspring, and it has been claimed that about 2.5% of these have an identical phenotype [65]. The transgenerational transmission of a defect induced by thalidomide is therefore a reasonable hypothesis. It is very *unlikely* that this could be due to mutagenic activity, because there is no obvious way that an induced mutation would occur, nor is there any evidence that thalidomide is mutagenic in various test systems [66]. The transgenerational transmission of the phenotype might, however, be explained as heritable epigenetic defect. Thalidomide could interact with a protein or cell receptor to induce a defect in the normal pattern of DNA methylation. It has been reported to affect or interact with DNA [67,68], but random interaction would not induce specific effects. It is conceivable, however, that it acts in conjunction with a sequence-specific DNA binding protein. The mechanism of action of thalidomide remains unknown, but evidence suggests that it can produce epigenetic and transgenerational effects in humans.

Similarly, isotretinoin (13-cis-retinoic-acid), a derivative of retinoic acid (RA) which is often used to treat severe acne and is also sometimes used as a chemotherapy medication for prevention and treatment of certain skin cancers, is such a strong teratogen that just a single dose taken by a pregnant woman may result in serious birth defects. When births occur, they are found to have approximately 30% rates of congenital malformation, versus a 3–5% baseline risk [69].

Retinoic acid (RA) mediates many of the functions of vitamin A, which regulates gene expression by activating intracellular RA receptors [70]. The functions of vitamin A are essential for immunological function, reproduction and embryonic development as shown by the impaired growth, susceptibility to infection and birth defects observed in populations receiving suboptimal vitamin A in their diet. It is now known that RA can influence gene expression and protein production in many ways [70]. Genes can respond to RA through a "direct" pathway; while others respond through "indirect" mechanisms. More than 500 genes have been put forward as regulatory targets [70]. Isotretinoin's exact mechanism of action is unknown, but it is known that, like RA, it alters DNA transcription [71]. It has also recently been shown to alter DNA methylation patterns, although it is not yet known if these are direct or indirect effects [72]. In any case, the gene expression changes cause decreased size and output of sebaceous glands, making the cells that are sloughed off less sticky, and therefore less able to form comedones. Isotretinoin noticeably reduces the production of sebum and shrinks the sebaceous glands, and stabilizes keratinization, preventing comedones from forming. Adverse drug reactions associated with isotretinoin include dryness of skin, lips and mucous membranes, infection of the cuticles, cheilitis, skin fragility and peeling, nose bleeds, dry eyes, conjunctivitis and other ocular problems, hyperlipidaemia, raised liver enzymes, alopecia, myalgia and/or arthralgia, headaches and intracranial hypertension, depression, psychosis, and other psychiatric disorders [73]. The following adverse effects have been reported to persist, even after discontinuing therapy, suggesting persistent (or perhaps slowly-reversing) gene expression changes and epigenetic effects: alopecia [73], arthralgias [74], ocular abnormalities [75,76], inflammatory bowel disease [77,78], keloids [79], osteopenia [80], hyperlipidemia [81], erectile dysfunction [82], and psychiatric

disturbances [83]. Isotretinoin is postulated to have complex effects on the brain and central nervous system. One study utilizing positron emission tomography (PET) showed functional brain imaging changes in treated patients [84].

Neuroleptics, SSRIs, ritalin, adderall: psychiatric drugs and cerebral gene expression

If we consider the brain as a massively parallel computer, the physical organization and synaptic connections of neural networks could be seen as the "hardware", while cerebral gene expression regulatory networks could be considered as the "software". Likewise, "brain damage" could result from damage to either the physical neural networks themselves, or damage to the software (viz. neural gene expression regulatory networks [85]) running on such "hardware".

One class of medication suspected of causing the latter form of damage is neuroleptics, which are used to treat symptoms of schizophrenia primarily by blocking dopamine receptors [86]. The long-term use of these drugs causes an iatrogenic disease termed "Tardive Dyskinesia" (TD), which refers to a variety of involuntary, repetitive movements such as grimacing, tongue protrusion, lip smacking, puckering and pursing of the lips, and rapid eye blinking [87]. Rapid twitching of the arms, legs, and trunk may also occur [87]. "Dyskinesia" refers to the involuntary movement, and "Tardive", means that the dyskinesia continues or appears even after the drugs are no longer taken, and is frequently irreversible. Despite the fact that TD has existed for over 50 years, its etiology is poorly understood. The most likely cause appears to be related to epigenetic damage to the system that uses and processes the neurotransmitter dopamine. Neuroleptics act primarily on the receptors for this neurotransmitter, and older neuroleptics, which have greater affinity for dopamine D2 receptors, are associated with high risk for TD: a rate of 5% per year, with rates plateauing after approximately 5–8 years.

The most compelling hypothesis for the etiology of TD is that it results from neuroleptic-induced dopamine supersensitivity in the nigrostriatal pathway, with gene expression of the D2 dopamine receptor being most affected [88]. Three lines of evidence support this hypothesis. Foremost, between-group comparisons show higher base rates of TD among those treated with conventional neuroleptics, as opposed to atypical neuroleptics. Secondly, within-subject data showing medication-related symptom changes are also consistent with this hypothesis. Finally, additional support from genetic polymorphism studies in encouraging, though not yet conclusive. The D2 hypersensitivity hypothesis is also supported by evidence of a dose-response relationship, withdrawal effects, studies on D2 agonists and antagonists, animal studies, and genetic polymorphism research [88]. Furthermore, numerous studies have shown that neuroleptics induce widespread global expression changes in genes other than dopamine receptors, the reversibility of which has yet to be determined [89–91]. Antipsychotics also cause hyperglycemia [92] and accelerate the development of diabetes [93]. Since diabetes is now considered to be a disease with a potentially large epigenetic component [6], and it has been shown that transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia [94], it is tempting to speculate that neuroleptics may accelerate the development of diabetes by an epigenetic mechanism.

Besides data from the aforementioned valproate and neuroleptics, increasing evidence from studies of narcotics and psychotropic drugs suggests that gene expression changes in neurons are mediated by epigenetic mechanisms that alter chromatin structure on specific gene promoters [95,96]. Recent findings from behavioral, molecular and bioinformatic approaches are being used to understand the complex epigenetic regulation of gene expression by

psychoactive compounds [21]. Most importantly, several psychotropics that are currently in clinical use for a variety of conditions also exhibit epigenetic effects in addition to their commonly-understood modes of action [37], suggesting at least some degree of overlap between narcotic and psychotropic drug action. Indeed, the gene expression effects of methylphenidate, a stimulant used to treat Attention Deficit Hyperactivity Disorder (ADHD), on molecules of neuronal signaling and neuroplasticity, including transcription factors, neuropeptides, and components of second messenger cascades, are quite similar those produced by cocaine and amphetamines [97,98]. Methylphenidate treatment seems to potentiate synaptic plasticity [99] and alters processing of incentive values [100]. Early exposure to methylphenidate causes behavioral changes that endure into adulthood, evidence of epigenetic effects [101–103]. Some changes, such as reduced sensitivity to cocaine, may be beneficial, whereas others, including increases in depressive-like signs, and reduced habituation, may be detrimental.

Besides stimulants, SSRI antidepressants have been shown to cause long-term alterations in gene expression, presumably resulting from chronic elevation of serotonin (5-HT) neurotransmission in the brain [104]. For example, chronic treatment with fluoxetine (Prozac) has been shown to cause persistent desensitization of 5HT1A receptors even after removal of the SSRI [105]. These long-term adaptive changes in 5-HT receptors, as well as more complex, global changes, are likely to be mediated through alterations of gene expression [106–110]. Some of these gene expression changes are a result of altered DNA structure caused by chromatin remodeling [111,112], specifically epigenetic modification of histones [113] and gene silencing by DNA methylation due to increased expression of the methyl binding proteins MeCP2 and MBD1 [114] Induction of the aforementioned methyl binding proteins was accompanied by enhanced HDAC2 mRNA synthesis, and decreased amounts of histone H3 were detected in three serotonin projection areas: the caudate-putamen, the frontal cortex, and the dentate gyrus of the hippocampus. Taken together, it appears that increased MDB1, MeCP2 and HDAC2 expression and recruitment to DNA plays a role in the regulation of histone acetylation and repression of gene expression is a generalized response to fluoxetine.

Imipramine, a tricyclic antidepressant, can also affect chromatin remodeling and gene expression by altering the expression of *Bdnf* transcript III and IV promoter regions. It also causes hyperacetylation of histone H3 at *Bdnf* promoters P3 and P4, which was associated with a downregulation of HDAC5. Because described gene expression changes are complex, and can involve persistent modifications of chromatin structure, it has been suggested that chronic antidepressant use can result in persistently altered cerebral gene expression leading to compromised catecholaminergic neurotransmission and neuroendocrine disturbances, such as decreased testosterone levels [115], reduced fertility [116], and persistent sexual dysfunction [117–120].

Chemotherapeutics – "Chemo brain", and Bystander effects

Cancer patients frequently complain of neurological side effects such as memory loss and cognitive dysfunction and sometimes seizures, vision loss, and dementia [121]. Until recently, these symptoms were attributed to fatigue, depression, and anxiety related to cancer diagnosis and treatment. But evidence is accumulating that these symptoms, commonly referred to as "Chemo Brain", may be a persistent side-effect of chemotherapy [122].

A recent study showed that three common chemotherapy drugs used to treat a wide range of cancers were more toxic to healthy brain cells than the cancer cells they were intended to treat [123]. A similar series of experiments in which mice were exposed to doses of 5-fluorouracil (5-FU) in amounts comparable to those used in cancer patients, showed that months after exposure, oligodendrocytes, and dividing precursor cells from which they are generated, underwent such extensive damage that after six months almost all of the cells had been destroyed. The 5-FU caused both acute CNS damage and a syndrome of progressively-worsening delayed damage to myelinated tracts, which was associated with altered gene expression [124]. These findings parallel observations in studies of cancer survivors with cognitive difficulties. MRI scans of these patients' brains revealed a condition similar to leukoencephalopathy. This demyelination can be associated with multiple neurological problems [125].

Side effects of chemotherapeutics also include increased incidence of cancers secondary to those being treated [126]. Since most chemotherapy drugs are genotoxic, it is quite likely that they cause epigenetic damage. In fact, it has been demonstrated that genotoxic carcinogens, in addition to exerting genotoxic effects, often cause epigenetic alterations [127]. For example, Tamoxifen is a non-steroidal anti-estrogen used for the treatment and prevention of breast cancer. It is also a potent hepatocarcinogen in rats, with both tumor-initiating and tumor-promoting properties. There is substantial evidence that the hepatic tumors in rats are initiated as a result of formation of tamoxifen-DNA adducts. Recently it was shown that the mechanism of tamoxifen-induced hepatocarcinogenesis also includes an epigenetic component. In rats fed tamoxifen in their diet, global liver DNA hypomethylation increased up to 200%. Protein expressions of maintenance (DNMT1) DNA methyltransferase and de novo DNA methyltransferases DNMT3a and DNMT3b were decreased. Likewise, trimethylation of histone H4 lysine 20 was significantly decreased [128]. Tamoxifen has also been shown to induce very rapid, irreversible epigenetic inactivation of estrogenic responses [129] permanent chromatin remodeling [130] and profound changes in microRNA expression [131]. This confirms that tamoxifen can cause permanent epigenetic modifications in human cells, but the importance of these findings to the etiology of tamoxifen-induced hepatocarcinogenesis needs to be explored.

General anesthetics (postoperative cognitive dysfunction and protein misfolding)

More than 100 million people undergo surgery every year, most of which is carried out under general anesthesia, with an inhaled anesthetic, such as isoflurane, halothane, or sevoflurane. Because of the improved safety of anesthesia through the use of advanced monitoring technology and training, increasing numbers of the elderly are safely enduring general anesthesia; even though the mechanism of action and the full extent of possible side effects have yet to be elucidated. These drugs clearly influence cognition, at least temporarily, in that the patient is made unconscious, unaware, insensate and amnesic for the duration of the surgery, and briefly thereafter. However, there is growing concern that anesthetics might affect a patient's cognitive abilities beyond the perioperative period, even permanently, and especially at the extremes of age.

Current evidence suggests that some inhaled anesthetics are capable of causing apoptosis [132], leading to a vicious cycle of apoptosis and amyloid beta-protein accumulation [133] neuronal damage [134] and durable cognitive dysfunction [135]. Potential mechanisms are varied but a recent study shows that anesthetics can enhance protein misfolding and aggregation. Thus, it has been proposed that anesthetic-induced neuronal injury could follow similar pathways as the neurodegenerative disorders, such as Alzheimer's or Parkinson's disease [136]. Interestingly, the age of Alzheimer's disease onset has been associated with previous surgery at odds ratios of up to 1.7 [137], which constitutes a worrisome

trend. It has been shown that general anesthetics can cause substantial changes in gene and protein expression [138,139]. For example, even brief exposure to isoflurane leads to widespread changes in genetic control in the amygdala 6 h after exposure [140]. Such gene expression changes have been found to persist up to days after exposure to the anesthetic has ceased [141], suggesting that recovery of the brain from anesthesia is considerably slower than generally recognized, and the persistence of effects caused by epigenetic mechanisms should be considered.

Synthetic estrogens and the Combined Oral Contraceptive Pill (COCP)

The Combined Oral Contraceptive Pill (COCP) is a combination of an estrogen and a progestin, taken by mouth to inhibit normal female fertility. Estrogen and progestin are both sex steroids, hormones that interact with receptors. Their effects are mediated by genomic mechanisms through nuclear receptors as well as by non-genomic mechanisms through membrane-associated receptors and signaling cascades. Fetal and neonatal exposure to a prototypical synthetic estrogen, diethylstilbestrol (DES), which was used to prevent miscarriages and other pregnancy complications between 1938 and 1971, is known to cause cancer in the male and female reproductive tracts later in life [142,143]. Many other adverse associations have been identified in DES-exposed women and their offspring, and animal studies have shown effects in the next generation (grandchildren), a clear demonstration of transgenerational epigenetic effects [144,145]. There is now significant evidence that that DES-induced abnormalities of reproductive organs are associated with altered expression levels of DNA methyltransferases and DNA methylation [146]. Estrogen was also recently shown to cause very rapid epigenetic changes in breast cancer cells [147], suggesting that steroid hormone mediated epigenetic regulation can affect gene expression immediately and long-term. COCP increases the risk of breast cancer by an average of 44% in pre-menopausal women who took, or were taking, oral contraceptives (OCs) prior to their first pregnancy, according to a comprehensive analysis of international studies conducted between 1980 and 2002 [148]. Of the 23 studies examined, 21 showed an increased risk of breast cancer with COCP use prior to a first pregnancy in pre-menopausal women. The study reinforces the 2005 classification of COCP as a Type 1 carcinogen in humans by the International Agency for Cancer Research.

Research is constantly being published regarding hormonal contraceptives and bone health, migraine headaches, depression, thrombosis risk, hypertension, cancer, weight gain, and obesity [149]. Since it is now well-established that estrogen can cause epigenetic changes, it is reasonable to design experiments that ask whether the side effects associated with the use of synthetic estrogens are fully reversible after cessation of the drug. For example it has been shown that the COCP can cause sexual dysfunction by elevating levels of Sex Hormone Binding Globulin (SHBG) [150]. SHBG binds to sex hormones, including testosterone, rendering them unavailable. Even after women stop taking the COCP, SHBG levels remain elevated and no reliable data exists to predict when they will diminish. Indeed, it has already been suggested that this is a persistent epigenetic effect on SHBG gene expression [151].

Chloroquine and fluoroquinolone antibiotics

The quinolones are a family of broad-spectrum antibiotics. They inhibit the bacterial DNA gyrase or the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription [148]. Eukaryotic cells do not contain DNA gyrase or topoisomerase IV, so it has been assumed that quinolones and fluoroquinolones have no effect on human cells, but they have been shown to inhibit eukaryotic DNA polymerase alpha and beta, and terminal deoxynu-

cleotidyl transferase [152], affect cell cycle progression and function of lymphocytes *in vitro* [153], and cause other genotoxic effects [154]. These agents have been associated with a diverse array of side-effects including hypoglycemia, hyperglycemia, dysglycemia, QTc prolongation, torsades des pointes, seizures, phototoxicity, tendon rupture, and pseudomembranous colitis [155]. Cases of persistent neuropathy resulting in paresthesias, hypoaesthesias, dysesthesias, and weakness are quite common [156]. Even more common are ruptures of the shoulder, hand, Achilles, or other tendons that require surgical repair or result in prolonged disability [157]. Interestingly, extensive changes in gene expression were found in articular cartilage of rats receiving the quinolone antibacterial agent ofloxacin, suggesting a potential epigenetic mechanism for the arthropathy caused by these agents [158]. It has also been documented that the incidence of hepatic and dysrhythmic cardiovascular events following use of fluoroquinolones is increased compared to controls, suggesting the possibility of persistent gene expression changes in the liver and heart [159].

Beta-blockers

Beta-blockers (β-blockers) are a class of drugs used to treat hypertension and manage cardiac arrhythmias and cardioprotection after myocardial infarction. Side-effects associated with their use include: bronchospasm, dyspnea, bradycardia, hypotension, heart failure, heart block, various psychiatric disorders, sexual dysfunction, and alteration of lipid and glucose metabolism [160]. The latter is particularly troublesome since recent studies have revealed that beta-blockers, especially when used in combination with diuretics, increase a patient's risk of developing diabetes [161,162]. Since diabetes is now considered to be a disease with a potentially large epigenetic component [6], and as previously mentioned, transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia [94], it is tempting to speculate that beta-blockers may accelerate the development of diabetes by an epigenetic mechanism.

Statins

The statins (or HMG-CoA reductase inhibitors) are a class of hypolipidemic drugs used to lower cholesterol levels in people with or at risk of cardiovascular disease. They lower cholesterol by inhibiting the enzyme HMG-CoA reductase, which is the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis. Inhibition of this enzyme in the liver stimulates the production of LDL receptors, presumably by an epigenetic mechanism, resulting in an increased clearance of low-density lipoprotein (LDL) from the bloodstream and a decrease in blood cholesterol levels [163]. Many patients on statin therapy suffer from myalgias, muscle cramps, and sometimes gastrointestinal or other symptoms [164]. Liver enzyme derangements and multiple other side-effects may also occur. The precise mechanism of muscle injury and other side effects is unknown, but it is known that statins cause extensive alterations in gene expression in target organs [165]. Decreased expression of the atrogin-1 gene by an epigenetic mechanism, is believed to be responsible for promoting muscle fiber damage [166,167]. It has also been proposed that mitochondrial impairment by statins leads to a mitochondrial calcium leak that directly interferes with the regulation of sarcoplasmic reticulum calcium cycling, without excluding a direct effect of statin on the sarcoplasmic reticulum [168]. Both mitochondrial and calcium impairments may account for the apoptotic process, oxidative stress, and muscle remodeling and degeneration.

Cox-2 inhibitors

A Cox-2 selective inhibitor is a form of Non-steroidal anti-inflammatory drug (NSAID) that directly targets Cox-2, an enzyme responsible for inflammation and pain [169]. Selectivity for Cox-2 reduces the risk of peptic ulceration, and is the main feature of celecoxib (Vioxx), rofecoxib and other members of this drug class. Cox-2-selectivity does not seem to affect other adverse-effects of NSAIDs, and epidemiological studies have shown that there is an increased risk of heart attack, thrombosis and stroke by a relative increase in thromboxane. Interestingly, there is some data suggesting that even after patients stop taking Vioxx, they still have a 74% higher stroke/heart attack risk [170]. What this means is the relative risk of a cardiovascular event with Vioxx even after the drug is stopped, is very similar to the risk while taking the drug. A persistent epigenetic effect on cardiovascular tissues is one possibility, since Cox-2 inhibitors have been shown to cause extensive gene expression changes [171,172].

Consequences of the hypothesis and discussion

Pharmaceutical drugs act on cellular processes or pathways to induce physiological changes. In addition to beneficial effects, most drugs carry risks for at least some side-effects. These side-effects are a consequence of conceptual reductionism in drug design and discovery, mainly due to a lack of knowledge about the pathophysiological pathways and gene regulatory networks they are acting on [173]. Moreover, we postulate that in addition to exerting effects by a purely traditional pharmacological mechanism, many pharmaceutical drugs also cause epigenetic changes that may or may not be beneficial. These effects can be divided into "direct" and "indirect" mechanisms (Fig. 1).

Above, we have provided examples of some drugs with a previously assumed mechanism of action (i.e. hydralazine) that have subsequently been shown to interfere directly with the normal controls of DNA methylation, resulting in aberrant gene expression. One way they do this is by interacting with specific proteins

Fig. 1. We postulate two types of epigenetic side-effects caused by pharmaceutical drugs, shown here in this schematic. These effects can be divided into "direct" and "indirect" mechanisms. Direct effects are caused by drugs that interfere directly with the normal controls of DNA and/or histone methylation, resulting in aberrant gene expression. For example by altering the action of DNA binding proteins, DNA methyltransferases, or histone demethylating enzymes etc. Other drugs are postulated to cause epigenetic changes by interaction with a cell surface receptor, enzyme, or other protein, which thereby alters expression of said receptors, and subsequently alters expression of growth factors, ion channels, structural molecules, or transcription factors and thereby alters cellular homeostasis. If chronic, this persistently altered cellular gestalt induces a secondary change in DNA methylation by a heretofore unknown cellular feedback mechanism – resulting in a changed epigenetic homeostasis. We hypothesize that cells are able to "sense" chronic changes in subcellular physiological processes, and subsequently adapt or imprint these alterations into DNA methylation patterns and/or histone and chromatin architecture.

which target particular DNA sequences, acting in concert with a DNA methyltransferase, or alternatively with a demethylating mechanism. This is a "direct" epigenetic effect (Fig. 1).

Other drugs (i.e. neuroleptics) appear to induce epigenetic changes by interaction of the drug with a cell surface receptor, enzyme, or other protein, which alters expression of said receptors, growth factors, ion channels, structural molecules, or transcription factors and which subsequently alters cellular homeostasis. If chronic, this persistently altered cellular gestalt induces a secondary change in DNA methylation by a heretofore unknown cellular feedback mechanism (Fig. 1) resulting in a changed epigenetic homeostasis. We hypothesize that cells are able to "sense" chronic changes in subcellular physiological processes, and subsequently adapt or imprint these alterations into DNA methylation patterns and/or histone and chromatin architecture. This would be termed an "indirect" epigenetic mechanism. One previously well-characterized example of such mediation is the induction of vitellogenin synthesis by estradiol in chicken liver. This is associated with the loss of methyl groups at specific sites in the estrogen response element of the vitellogenin gene [174]. Pharmaceutical drugs might act through a similar pathway, but the methylation or demethylation of genes would be disrupted in a drug-specific fashion, and this in turn would produce drug-specific side-effects.

Epigenomic screening of drugs would expand our understanding of their mechanism of action, which would potentially improve their clinical utility. As our understanding of how cellular signaling circuitries feeds back into epigenomic regulation (and vice-versa) expands, information on DNA methylation effects of drugs will provide us with missing links in our understanding of the cellular mechanisms of these drugs and pharmacology as a whole.

We strongly propose that high-throughput whole-epigenomic screens using methylated/unmethylated CG arrays and other whole-genome mapping approaches should be utilized to identify the potential impact of drugs in clinical development, as well as drugs already in clinical practice, on DNA methylation patterns [35]. Epigenomic effects of drugs would at the very least increase our understanding of their mechanism of action. In difference from the traditional candidate gene approaches used in the past, current epigenomic approaches would allow for a non-biased approach and might unravel unpredicted effects of drugs. We certainly need to improve our understanding of the molecular mechanisms underlying complex cellular processes and consider each drug target in its full epigenetic context. We suggest the best strategies might be those that combine computational and experimental techniques, and a systems pathology approach will ultimately lead to a better comprehension of the molecular effects of pharmaceuticals. These new approaches, which have been termed "pharmacoepigenomics" or "toxicoepigenomics" allow for the discovery of potential untoward effects of drugs early in the drug development program and might save significant time, effort, money, and even lives by eliminating potentially toxic drugs from the development pipeline [175].

DNA methylation effects might be extremely teratogenic during embryogenesis (i.e. thalidomide and isotretinoin, as discussed above) and might have potential carcinogenic effects. It is well known that the pattern of DNA methylation is heritable, both through mitosis and from one generation to the next [176]. The methylation program for development is reset in germ line cells, prior to, during or after meiosis. There is some information about the specific controls of DNA methylation in the germ line, and during early embryogenesis, and genomic imprinting is certainly associated with differences in DNA methylation in male and female gametes, and is a reversible change [177]. There are also defects in genomic imprinting, such as the Prader Willi syndrome, which produce characteristic phenotypes [178]. Thus a strong case can be made that induced abnormalities in DNA methylation caused
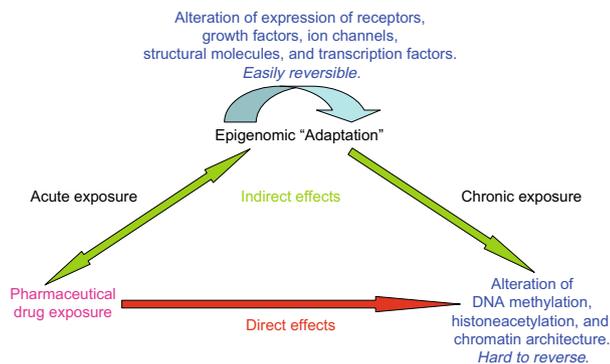
by pharmaceutical drugs can produce defects in subsequent generations. It would be alarming if the worldwide increases in diabetes and obesity, which have been postulated to be epigenetic [179,180], were actually being accelerated by the increasing use of pharmaceutical agents.

We have already discussed the potential transgenerational effects of thalidomide, and it appears as though some behavioral effects of antidepressants might be transgenerationally inherited, at least in rodents, since maternal exposure to fluoxetine impairs sexual motivation in adult male mice [181]. There may even be conflicting or synergistic consequences of maternal and pharmacological influences on epigenetic changes in behavior, and the interaction of such factors may be complex. In any case, we can postulate a link between the effect of a drug on somatic cells, and the same effect of the drug on germ line cells. For example, if the pharmaceutical causes DNA methylation changes in somatic cells by a "direct" effect, it might also target the same pathway in germ line cells. Or it might interfere in an "indirect' way with a receptor common to both types of cell. More generally, it could target any mechanism which influences the pattern of DNA methylation or chromatin architecture in a particular region of the genome. In this way, the methylation of the same DNA sequence would be altered in somatic cells to produce a side-effect, and also in germ line cells of the developing fetus to produce the same or a similar change in DNA methylation. If this is eventually transmitted to a subsequent generation, the same type of defect might be seen. As discussed in the introduction, this type of event is referred to as the transgenerational transmission of an epigenetic defect. Experimental techniques using human embryonic stem cells and *in vitro* differentiation systems could be used to assess such developmental epigenetic effects [182,183].

On a more positive note, besides detecting potential side-effects, epigenomic screens might identify potential epigenomic drugs that might be of use in treating some diseases. Also, the DNA methylation inhibitory effects of drugs might have some therapeutic advantage in addition to the untoward effects. For example, valproate, hydralazine and procainamide might be utilized to induce gene expression in cancers where induction of a methylated gene might be of benefit [184,185].

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