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Perspective

DNA-Damaging Agents in Cancer Chemotherapy: Serendipity and Chemical Biology

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DNA-damaging agents have a long history of use in cancer chemotherapy. The full extent of their cellular mechanisms, which is essential to balance efficacy and toxicity, is often unclear. In addition, the use of many anticancer drugs is limited by dose-limiting toxicities as well as the development of drug resistance. Novel anticancer compounds are continually being developed in the hopes of addressing these limitations; however, it is essential to be able to evaluate these compounds for their mechanisms of action. This review covers the current DNA-damaging agents used in the clinic, discusses their limitations, and describes the use of chemical genomics to uncover new information about the DNA damage response network and to evaluate novel DNA-damaging compounds.

Although considered by some to be a modern disease, cancer in humans has been documented for millennia (for review, see David and Zimmerman, 2010). Currently, cancer accounts for 7 to 8 million deaths (13% of all deaths) worldwide (World Health Organization, 2012). Despite repeated campaigns to defeat cancer, such as Nixon's War on Cancer, all have failed, because cancer is not a single disease. In fact, it is a collection of highly complex diseases characterized by unregulated cell proliferation that can arise from contributions from numerous different factors, including genetic and environmental.

The treatment of cancer is still largely based on the use of chemotherapeutic drugs to eliminate cancer cells, reduce tumor growth, and alleviate pain. The first widely used cancer drugs were discovered in the 1940s as a result of studying victims of chemical warfare during World Wars I and II (for review, see Chabner and Roberts, 2005). Soldiers exposed to sulfur mustard gas were found to have depleted bone marrow and reduced lymph nodes (Krumbhaar and Krumbhaar, 1919). Alfred Gilman and Louis Goodman began testing more stable nitrogen mustard compounds, such as bis and tris β-chloroethyl amines, and found that they caused tumor regressions in mice with transplanted lymphoid tumors (Gilman, 1963; Gilman and Philips, 1946). Next, they treated a patient with late-stage non-Hodgkin's lymphoma with tris β-chloroethyl amine and found that the tumor subsided (Goodman et al., 1946). Subsequent testing of β-chloroethyl amines in 67 patients with non-Hodgkin's lymphoma and leukemia revealed marked tumor regression (Goodman et al., 1946). It was later noted that these remissions were short-lived, with resistance to the compounds developing rapidly; however, the idea that tumors could be cured, if only temporarily, ushered in an era of widespread research into discovering and characterizing cancer therapeutics.

Around the same time (1946–1948), Sidney Farber was studying the effects of folic acid in leukemia patients. He discovered that, when folic acid was administered to these patients, it led to increased proliferation of acute lymphoblastic leukemia cells (Farber, 1949). Folic acid deficiencies were identified in patients with megaloblastic anemia, and its administration was found to stimulate bone marrow maturation and growth (Wills et al., 1937). Farber's observation led to collaborations with Yellapragada Subbarao to develop folate analogs that could chemically block folic acid and hence inhibit the production of abnormal bone marrow associated with leukemia. This was one of the first examples of rational drug design, as opposed to serendipitous discovery. One of these folate analogs, aminopterin, was administered to children with acute lymphoblastic leukemia and led to successful remissions (Farber and Diamond, 1948). Though the remissions were brief, it was clear that antifolates had potential as anticancer compounds. Another folate analog with less toxicity than aminopterin, methotrexate (amethopterin) was one of the first drugs to cure a solid tumor (choriocarcinoma) in the 1950s (Li et al., 1958).

It took a decade to identify what these two compounds had in common: both nitrogen mustards and folate antagonists are effective at killing cancer cells due to their DNA-damaging properties. DNA integrity is critical for proper cellular function and proliferation. High levels of damage to DNA are detected by cell-cycle checkpoint proteins, whose activation induces cell-cycle arrest to prevent the transmission of damaged DNA during mitosis. DNA lesions that occur during the S phase of the cell cycle block replication fork progression and can lead to replication-associated DNA double-strand breaks (DSBs), which are among the most toxic of all DNA lesions. If the damaged DNA cannot be properly repaired, cell death may result. Cancer cells typically have relaxed DNA damage-sensing/repair capabilities and, more importantly, they are capable of ignoring cell-cycle checkpoints, allowing the cells to achieve high proliferation rates; this also makes them more susceptible to DNA damage, since replicating damaged DNA increases the likelihood of cell death. The concept of aiming at DNA as a target for anticancer drugs inspired the development of numerous anticancer compounds, such as cisplatin, doxorubicin, 5-fluorouracil, etoposide, and gemcitabine.

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Table 1. Properties of DNA-Damaging Compounds Used in the Treatment of Cancer

Drug	Mode of Action	Major Side Effects	Mechanisms of Resistance
Cisplatin	DNA crosslinker	nephrotoxicity; neurotoxicity; ototoxicity	decreased uptake; increased efflux; enhanced replication bypass; increased DNA repair capacity; increased DNA damage tolerance; failure of death pathways
Carboplatin	DNA crosslinker	myelosuppression	
Oxaliplatin	DNA crosslinker	neurotoxicity; pulmonary toxicity; hepatotoxicity	
Methotrexate	prevents DNA synthesis by inhibiting dihydrofolate reductase (DHFR)	myelosuppression; pulmonary toxicity; gastrointestinal toxicity; hepatotoxicity; nephrotoxicity; neurotoxicity	increased DHFR expression; mutations in folate transporter genes
Doxorubicin	topoisomerase II poison	cardiotoxicity; myelosuppression; neurotoxicity	P-glycoprotein-mediated MDR; decreased topoisomerase II expression; enhanced DNA repair; decreased activity due to increased glutathione levels
Daunorubicin	topoisomerase II poison	cardiotoxicity; myelosuppression	

DNA-Reactive Agents

In the 1960s and 1970s, there was a surge of interest in developing anticancer compounds that react chemically with DNA. These included compounds that directly modify DNA bases, intercalate between bases, or form crosslinks in DNA. The nitrogen mustards studied by Goodman and Gilman act by directly alkylating DNA on purine bases, leading to stalled replication fork progression and subsequent cell death via apoptosis. Derivatives of nitrogen mustards were developed, including the DNA alkylators cyclophosphamide, chlorambucil, and melphalan, all of which are currently used in clinical therapeutics. Other examples of DNA-alkylating agents used in cancer treatment include nitrosoureas (e.g., carmustine, lomustine, and semustine) and triazenes (e.g., dacarbazine and temozolomide). Natural products which alkylate DNA bases were also discovered around this time, such as mitomycin C and streptozotocin. These compounds and several of the alkylators mentioned above crosslink DNA on opposite strands of the double helix (interstrand crosslinks), resulting in a more potent effect against cancer cells compared to monofunctional alkylation. For example, carmustine (*N*,*N*′-bis(2-chloroethyl)-nitrosourea) binds to the N1 of guanine on one DNA strand and the N3 of cytosine of the opposite strand to form interstrand crosslinks, which block DNA replication and can cause cell death if not repaired (Fischhaber et al., 1999).

The discovery of the alkylating-like platinum agents had a significant positive impact on anticancer drug research. Cis-diamminedichloroplatinum(II) (cisplatin), was discovered by accident in the 1960s, when a magnetic field generated by platinum electrodes was shown to block *E. coli* cell division (Rosenberg et al., 1965; Table 1). Cisplatin, as its name implies, contains a platinum core with two chloride leaving groups and two amine nonleaving groups (Figure 1). After cell entry, aquation of the chloride groups allows the platinum to bind guanine residues and, to a lesser extent, adenine residues to form adducts on DNA. When two platinum adducts form on adjacent bases on the same DNA strand, they form intrastrand crosslinks (Kelland, 2007; Siddik, 2003). The structures of these platinum-DNA adducts have been solved at atomic resolution using X-ray crystallography and nuclear magnetic resonance (Huang et al., 1995; Takahara et al., 1995). Inspired by the efficacy of cisplatin, platinum-based analogs have been developed, including carboplatin and oxaliplatin, which also act by forming DNA crosslinks but have different pharmacological properties, decreased side effects, and increased efficacy against different tumors (Wheate et al., 2010; Table 1). In particular, platinum compounds have been very successful in the treatment of solid tumors. Indeed, cisplatin therapy can cure over 90% of all testicular cancer cases and also has good efficacy in the treatment of ovarian, bladder, head and neck, and cervical cancers (Kelland, 2007). Current efforts to develop cisplatin analogs are aimed at reducing toxicity to nontargeted tissues, which results in dose-limiting toxicities, such as nephrotoxicity and neurotoxicity. The spectrum of different platinum compounds under development is broad, and platinum compounds have also encouraged the synthesis and testing of other metal-containing compounds for use in chemotherapy (Bruijnincx and Sadler, 2008; Köpf-Maier, 1994).

Antimetabolites

Antimetabolites represent a class of anticancer drugs that mimic normal cellular molecules and consequently interfere with DNA replication. Many of these compounds are DNA antagonists that exert their activity by blocking nucleotide metabolism pathways. Examples of widely used antimetabolite anticancer compounds include the pyrimidine analogs 5-fluorouracil (5-FU), capecitabine, floxuridine, and gemcitabine, and the purine analogs 6-mercaptopurine, 8-azaguanine, fludarabine, and cladribine. The incorporation of purine and pyrimidine analogs into DNA during the S phase of the cell cycle prevents proper nucleobase addition, causing DNA replication to fail. For example, 5-FU can be incorporated into DNA and RNA in place of thymine or uracil, respectively. Because 5-FU contains a fluoride atom at the 5-carbon position on the ring, it prevents the addition of the next nucleobase on the strand, therefore terminating chain elongation, which induces apoptosis (Parker and Cheng, 1990). In addition to nucleobase analogs, other antimetabolites inhibit enzymes important for DNA synthesis. Methotrexate, aminopterin, and newer antifolates, such as pemetrexed, inhibit the dihydrofolate reductase enzyme to block the synthesis of nucleotides. Another antifolate, ralitrexed, directly inhibits thymidylate synthase. Methotrexate, the primary antifolate used in chemotherapy, displays a broad range of antitumor activities against

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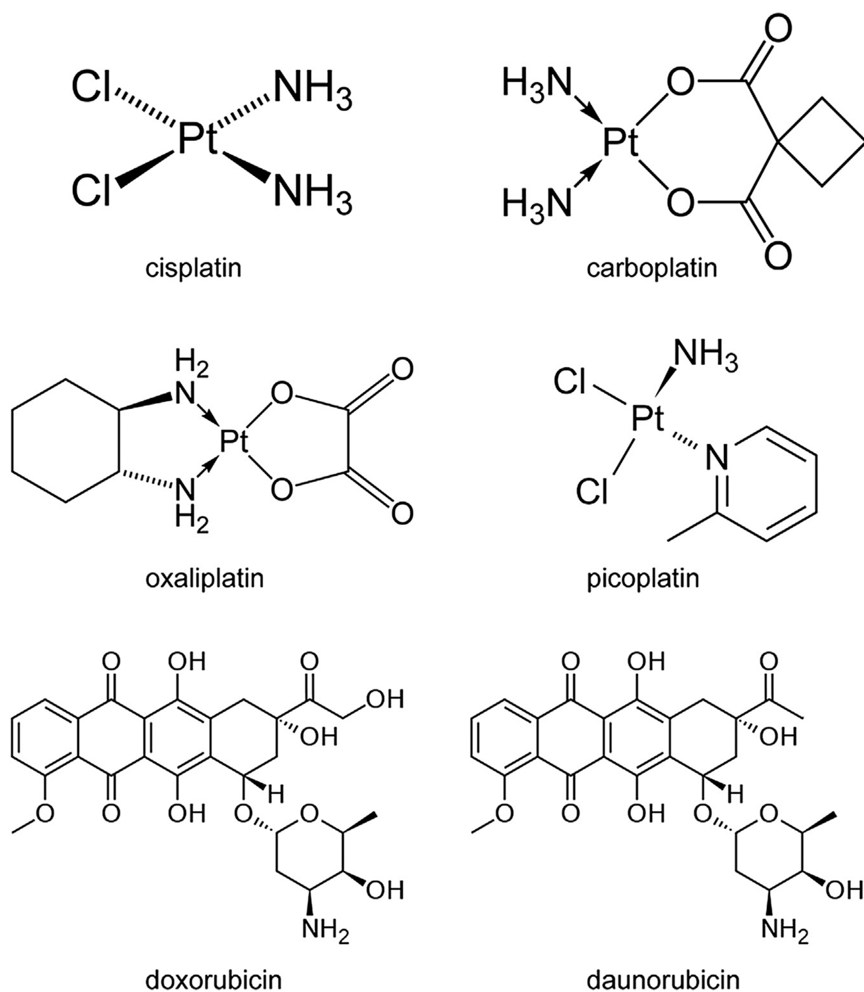

Figure 1. Structures of Selected DNA-Damaging Anticancer Compounds
Cisplatin, carboplatin, oxaliplatin, and picoplatin are platinum-based compounds. Doxorubicin and daunorubicin are anthracyclines.

breast, ovarian, bladder, and head and neck cancers (Bertino and Cronstein, 2000; Table 1).

Topoisomerase Poisons

A mechanistically distinct way to interfere with normal DNA function is to target protein-DNA complexes. The assembly of proteins onto DNA is crucial for many DNA processes, including transcription, replication, recombination, and repair. Therefore, it is not surprising that many DNA-active drugs act by interfering with DNA-protein binding. Topoisomerases are a class of enzymes responsible for releasing the torsional strain of the DNA double helix. Topoisomerase I allows the passage of a single DNA strand through a transient single-strand break created in the complementary strand of the double helix. Topoisomerase II cuts both strands of the double helix to allow the passage of an intact helix to unwind supercoiled DNA. Topoisomerase poisons trap the DNA-enzyme intermediate as a complex, preventing religation of the break, inhibiting replication fork progression, and causing toxic DSBs (for review, see Froelich-Ammon and Osheroff, 1995). Initial insights into how these inhibitors work came from plant analogs developed from podophyllotoxin and its derivatives, such as etoposide and teniposide, which were found to have antineoplastic effects. Interestingly, it was found that DNA strand breaks caused by etoposide did not form when etoposide was incubated with purified DNA (Wozniak and Ross, 1983). It was soon discovered that etoposide binds to the topoisomerase II-DNA complex (Chen et al., 1984). The cellular levels of topoisomerase II determine the efficacy of etoposide as a cytotoxic agent, with higher levels leading to greater efficacy (Burgess et al., 2008). This correlation can be used to inform and design topoisomerase II-mediated chemotherapy. Another plant-produced product, camptothecin, was found to be a topoisomerase I poison (Hsiang et al., 1985). As with etoposide, camptothecin does not bind the enzyme or DNA alone but rather binds to the DNA-topoisomerase complex to inhibit strand religation (Hertzberg et al., 1989).

The anthracycline antibiotics are a group of antineoplastic agents that, like etoposide, poison topoisomerase II, but they have additional antitumor mechanisms, including the ability to intercalate into DNA (Cutts et al., 2005; Table 1). The anthracyclines doxorubicin and daunorubicin (Figure 1) (derived from *Streptomyces peucetius*) are widely used to treat breast cancer, small-cell lung tumors, soft tissue sarcomas and lymphomas, and acute lymphoblastic or myeloblastic leukemias (Minotti et al., 2004). These compounds and the newer anthracyclines epirubicin and idarubicin have become mainstays of cancer chemotherapy. Anthracyclines can be extremely cytotoxic, likely owing to their multiple mechanisms of action in addition to binding the DNA-topoisomerase complex. Anthracyclines are also able to intercalate into DNA, generate free radicals, bind and alkylate DNA, crosslink DNA, interfere with helicase activity, and induce apoptosis (for review, see Minotti et al., 2004). Among their more significant toxic side effects is their well-documented cardiotoxicity (for review, see Olson and Mushlin, 1990).

Limitations of Current Chemotherapeutics

In describing the limitations of anticancer treatments, we will focus on two of the most successful antineoplastic compounds, cisplatin and doxorubicin, as exemplar agents.

A primary cause of failure of anticancer treatments is the intrinsic or acquired resistance of a tumor to the drug, which often leads to disease reoccurrence (Sawicka et al., 2004). This was initially characterized in the early studies of the nitrogen mustards: after tumors receded, they would recur and

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subsequently become resistant to further treatment. Resistance to anticancer compounds can arise in various ways, and understanding these mechanisms can help inform new strategies of cancer treatment. In many cases, cells manifest multidrug resistance by reducing drug uptake and/or increasing drug efflux through modulation of the expression or activity of drug pumps, such as P-glycoprotein and other multidrug resistance (MDR) transporters in the ATP-binding cassette family (Gottesman, 2002). In cases where the drug has a specific target, such as with the antifolates, loss of a cell surface receptor or mutation of the specific drug target (e.g., by gene amplification in the case of dihydrofolate reductase) can cause resistance. Resistance to the anticancer drug cisplatin can occur as a result of increased levels of drug detoxification by boosting of the production of cellular thiols, enhanced replication bypass of platinum-DNA adducts, changes in levels of regulatory proteins, increased DNA repair capacity, increased DNA damage tolerance, and the failure of cell death pathways (Wang and Lippard, 2005). Doxorubicin resistance can arise from alterations in DNA damage-sensing and repair capacities and can also arise from decreased topoisomerase II expression and/or catalytic activity. Increased expression of antioxidants that increase glutathione peroxidase activity also decreases doxorubicin efficacy (Sawicka et al., 2004). Specific drug resistance can, in some cases, be addressed by combination treatments with compounds that act through different mechanisms of action.

Another limitation in the use of anticancer compounds arises from adverse toxicity to nontargeted tissues. Because most anticancer drugs were discovered based on their efficacy against cancer cells, little attention was initially given to their effects on other tissues. One major side effect of cisplatin is nephrotoxicity (Yao et al., 2007). Although the proximal tubule cells of the kidney are quiescent, they are selectively damaged by cisplatin. Mechanisms that have been suggested to explain toxicity to these cells include activation of mitogen-activated protein kinases, reactive oxygen species, and stimulation of inflammation and fibrogenesis (Yao et al., 2007). Another significant dose-limiting side effect of cisplatin is toxicity to the brain, where cisplatin use can lead to tinnitus, high-frequency hearing loss, and peripheral neuropathies, including loss of vibration sense, paresthesia, and weakness (Wang and Lippard, 2005). Platinum-based compounds preferentially enter the dorsal root ganglia and peripheral nerves and do not readily penetrate the blood-brain barrier (BBB). Cisplatin will bind to DNA and form adducts in dorsal root ganglia neurons, leading to apoptosis of the neurons. The mechanism by which the platinum-DNA adducts lead to neuronal apoptosis is not fully understood. Interestingly, because of their inability to traverse the BBB, platinum-based compounds can be delivered directly to the central nervous system to treat brain tumors (Olivi et al., 1993).

The main limitation to doxorubicin use is cardiotoxicity, including cardiomyopathy and congestive heart failure (CHF) (Minotti et al., 2004; Olson and Mushlin, 1990). Swain et al. (2003) analyzed 630 patients with breast carcinoma or small-cell lung carcinoma and found that ~26% of patients experienced doxorubicin-related CHF at a dose of 550 mg/m^2. Several mechanisms have been proposed to explain the particular sensitivity of the heart to doxorubicin-mediated toxicity. A widely accepted explanation is that oxidative stress induced by intramyocardial production of reactive oxygen species following doxorubicin treatment produces cardiotoxic effects (Šimůnek et al., 2009). Another is that the heart is susceptible to the anthracyclines, owing to its elevated levels of mitochondria activity, because doxorubicin binds to cardiolipin within the mitochondrial inner membrane (Berthiaume and Wallace, 2007). Additional explanations for doxorubicin toxicity include inhibition of nucleic acid and protein synthesis, release of vasoactive amines, alterations in adrenergic function and adenylate cyclase activity, changes in calcium transport, and alterations in cellular iron metabolism (Carvalho et al., 2009). Another organ targeted by doxorubicin is the brain, despite the drug not being able to cross the blood brain barrier. Studies have suggested that tumor necrosis factor-α (TNF-α) is responsible for this toxicity (Tangpong et al., 2006). Doxorubicin increases the production of TNF-α, which in turn increases the production of inflammatory cytokines by microglial cells in the brain. Drug-induced oxidative stress has also been linked to doxorubicin-mediated toxicity of the liver (Kalender et al., 2005).

While the mechanisms that underlie these side effects have been studied for years, our understanding remains incomplete. The severity of these side effects can be reduced by using combination therapies that have the effect of minimizing the overall dose of each single agent. In addition, synergistic combinations with nonoverlapping toxicities can reduce side effects. Timing of combinations can also be exploited; for example, patients undergoing doxorubicin therapy are often pretreated with tamoxifen to reduce the level of toxic metabolites derived from doxorubicin (Sawicka et al., 2004). While these and other clinical strategies can certainly improve outcomes, new therapies and a better understanding of traditional therapies will be invaluable.

New Designs for Chemotherapeutics

A common theme in drug discovery and development is to address limitations in current anticancer therapies by designing novel compounds with mechanisms that are based on successful drugs. Thousands of cisplatin analogs have been synthesized with the goals of (1) reducing toxicity to patients, (2) overcoming tumor resistance, and (3) increasing the range of antitumor activity. Early work in the design of novel platinum-based anticancer drugs focused on developing compounds through the modification of substituents surrounding the cisplatin core (Hambley, 1997; Figure 1). An early, clinically successful cisplatin analog, carboplatin, was developed by replacing the chloride leaving groups with a more stable bidentate dicarboxylate ligand (Figure 1). Carboplatin treatment is less nephrotoxic and less neurotoxic; however, it can lead to myelosuppression (Kelland, 2007). It also requires a higher dosage for efficacy compared to cisplatin. Another cisplatin analog, oxaliplatin, contains a diaminocyclohexane carrier as the nonleaving group. Oxaliplatin was shown to exhibit a different pattern of sensitivity against the National Cancer Institute-60 panel of tumor cell lines, which may be related to its ability to form crosslink patterns distinct from cisplatin (Rixe et al., 1996). Interestingly, oxaliplatin damage does not induce expression of genes involved in mismatch repair (Raymond et al., 2002). Picoplatin was designed to increase the steric bulk around the platinum core in an effort to reduce thiol-mediated inactivation. Picoplatin was found to have activity in cells resistant to cisplatin and to have antitumor activity in vivo

(Holford et al., 1998). Although picoplatin did not meet its primary endpoint of overall survival in Phase III trials for cisplatin-resistant small-cell lung cancer, it is currently in Phase II trials for metastatic colorectal cancer (Ciuleanu et al., 2010).

While these approaches have led to several compounds currently in clinical trials, because of their structural similarities to currently used platinum drugs, it is more likely that these compounds will offer incremental versus radical improvements over existing drugs. Approaches to platinum-based compound design that address the issue of drug resistance by maintaining antitumor activity but that are not constrained to adhering to all of the structure-activity features of cisplatin and its analogs, have significant potential. The design of compounds with biologically active carrier ligands has paved the way for platinum-intercalator complexes, in which the carrier group functions independent of the platinum. To date, compounds have been generated in which cisplatin is attached to DNA-intercalators, such as acridine orange, chloroquine, and ethidium bromide, essentially bringing the platinum to its site of action (Baruah et al., 2004). In addition to DNA-intercalators, "hybrid drugs," in which the platinum moiety is attached to doxorubicin or to estrogen analogs, have been tested and found to have increased efficacy against cisplatin-resistant tumors (Gagnon et al., 2004; Zunino et al., 1986).

The fact that cancer chemotherapy is limited by drug toxicity and resistance highlights the need to better understand drug mode of action within the cell. In addition, there is a clear need for novel compounds that act through different and/or complementary mechanisms, which can be combined with existing agents to overcome resistance. These novel compounds also need to be evaluated to understand their mechanisms of action. Unbiased methods to examine drug function can lead to the development of better anticancer drugs and treatment regimens. Below, we describe recent efforts and important insights that have come from such approaches.

Chemical Genomic Screening to Understand Drug Mechanism of Action

Since the completion of the human genome sequence in 2003, we have amassed a wealth of structural and functional information about the human genome and proteome. Until quite recently, much of this information had not been utilized in the development of new therapies. This is now changing, with recent advances in molecular biology, genetic engineering, and genome-scale screening providing powerful new technologies for identifying drug targets and understanding drug mechanism of action. Within the human genome, it is estimated that approximately 3,000 human genes are "druggable," defined by the potential ability of their protein products to bind drug-like chemical entities (Hopkins and Groom, 2002). However, less than half of the proteins expressed by the human genome are functionally characterized, suggesting that this number is an underestimate. Furthermore, this characterization is a starting point; it is also possible that the number of potential targets could be larger than the number of genes in the genome, for example, posttranslationally modified proteins or splice variants may be specifically druggable. To compile a comprehensive understanding of a drug's cellular actions, it would be ideal to identify all primary and secondary targets of a drug. To this end, tools need to be developed that are rapid, cost-efficient, and can be used to study all cellular proteins (and other macromolecules) with different types of small molecule drugs or probes. Chemical genomics is one such approach, which employs small molecules to explore gene function and to identify potential drug targets. An early example of the power of chemical genetics was the characterization of the protein tubulin as "colchicine-binding protein"; this discovery was made a decade before the tubulin gene was sequenced and 50 years before the term "chemical biology" was coined (Taylor, 1965). The complete sequence of the genome of human as well as other organisms has provided an invaluable "parts list" of potential targets. Below, we describe several comprehensive screening approaches to use that gene list to guide drug discovery.

Chemical Genomic Screens in Yeast

The budding yeast *Saccharomyces cerevisiae* has been the benchmark organism for the development, testing, and application of genomic technologies. It is also an ideal model organism for the development of high-throughput genomic screens. The first eukaryote to have its genome sequence completed, *S. cerevisiae* has a well-characterized genome and proteome, a rapid generation time, is inexpensive to cultivate, and is highly amenable to genetic manipulations, such as gene deletion and dosage level variation (Botstein et al., 1997). A great resource for the development of systematic screening technology in *S. cerevisiae* is the yeast knockout collection (YKO). This is a complete deletion set of haploid strains and heterozygous and homozygous diploid strains, in which each open reading frame in the yeast genome has been precisely deleted from start to stop codon and replaced with a *kanMX*-dominant drug resistance cassette (Giaever et al., 2002; Winzeler et al., 1999). The cassette contains two unique 20-nucleotide sequences, which act as barcodes for identifying each deletion strain. These barcodes are flanked by common primer sequences, allowing for PCR amplification of barcodes and subsequent hybridization to a DNA barcode microarray (Pierce et al., 2006) or next-generation sequencing (Smith et al., 2009) to identify strains in a mixed population of deletion strains. Therefore, the YKO collection can be pooled and grown in parallel and the relative abundance of each strain can be determined by the abundance of each barcode.

Indeed, the YKO collection presents an ideal resource for competitive growth assays that allow the systematic evaluation of growth of the deletion mutants in different conditions. By growing the strains in the presence of drug, one can identify strains that confer growth advantages or disadvantages to the drug (Figure 2). This pooled approach allows for a rapid method of identifying growth effects using an unbiased, miniaturized approach (Hoon et al., 2008).

One of the first chemical genomic screens developed using the YKO collection is based on the concept of haploinsufficiency, where a diploid cell bearing one single copy of a given gene grows indistinguishably from wild-type, except in conditions that require full protein function or activity. The assay, known as drug-induced haploinsufficiency profiling (HIP) is based on the observation that a strain containing a heterozygous deletion in an essential gene encoding the drug target results in sensitization of that strain to the drug (Giaever et al., 1999, 2004; Lum et al., 2004). Therefore, in a pooled culture, that strain would

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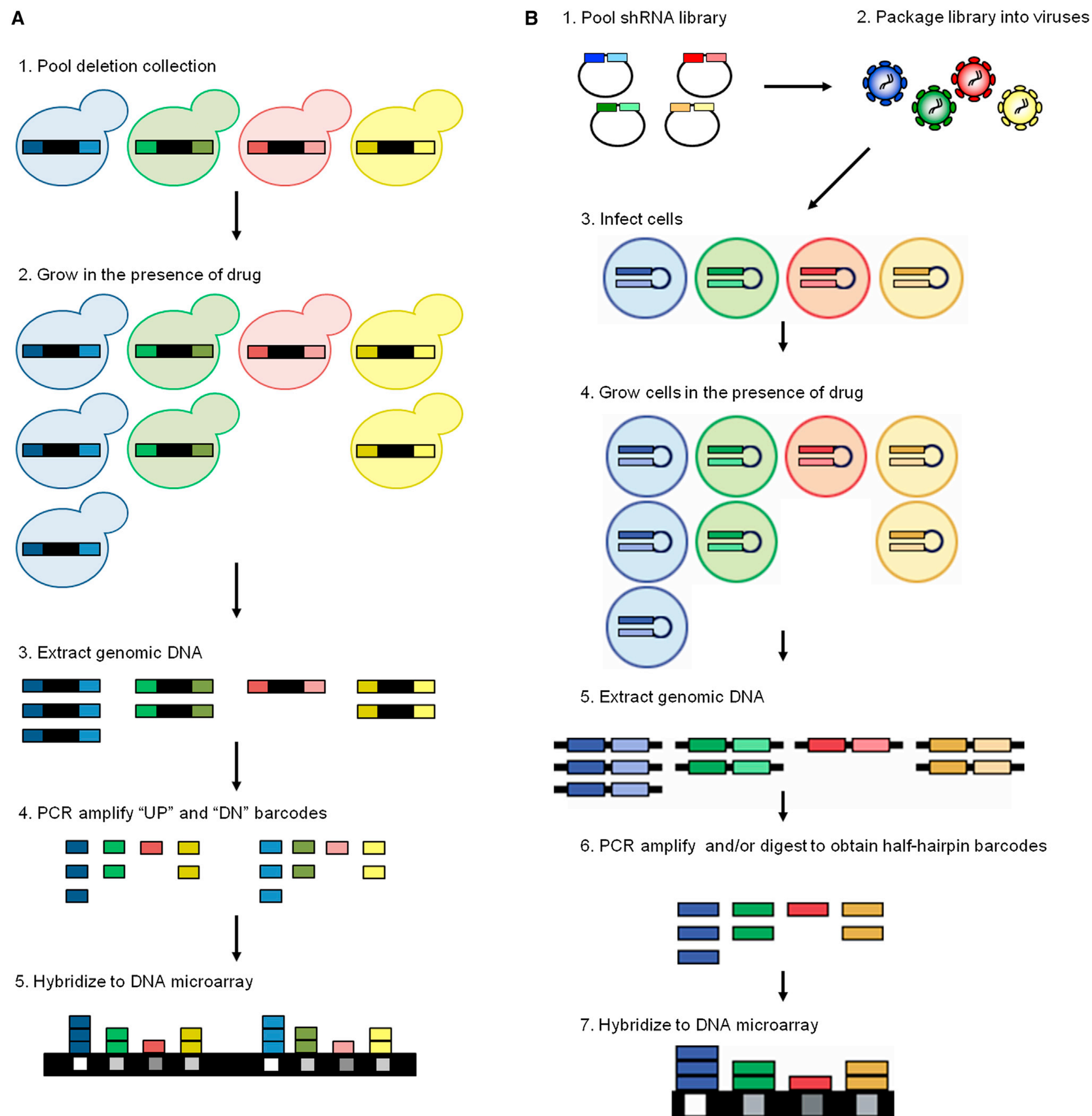

Figure 2. Chemical Genomic Screens

(A) The HIPHOP assay in yeast. (1) The YKO collection is pooled and (2) grown in the presence of a compound. (3) Genomic DNA is extracted from the pool and (4) DNA barcodes are PCR amplified. (5) The barcodes are hybridized to an Affymetrix TAG4 microarray. The signal intensity from the microarray is compared to an untreated control, and the relative intensity represents relative abundance of the strain in the pool.

(B) An shRNA-based loss-of-function screen. (1) A pooled shRNA library is (2) packaged into retro- or lentiviruses. (3) The virus pool is used to infect cultured cells, which are (4) grown in the presence of drug. (5) Genomic DNA is then extracted from the cells, and (6) hairpin sequences are amplified by PCR and/or digested to produce half-hairpin barcodes. (7) The barcodes are hybridized to a DNA microarray, and the signal intensity obtained on each probe is analyzed to find the relative abundance (compared to a no-drug control) of each shRNA in the population.

have a reduced fitness that can be quantified. The HIP assay has the ability to identify direct targets of compounds and proteins that may act in the same pathway as the target. In numerous studies, this assay has proven its ability to identify targets of well-known and novel compounds (Hillenmeyer et al., 2008; Smith et al., 2009; St Onge et al., 2007). For example, Giaever et al. (2004) demonstrated the power of the HIP assay for drug target identification through screens of ten diverse compounds,

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which included several anticancer compounds. In the screen for methotrexate-sensitive mutants, the most highly sensitive strain was that containing a heterozygous deletion for the yeast dihydrofolate reductase gene, *DFR1*. The HIP screen is also able to uncover additional pathways, through which some compounds act. The antimetabolite anticancer agent 5-FU is known to act through inhibition of thymidylate synthase. In HIP screens of this compound, genes that confer sensitivity were those involved in ribosome biogenesis and ribosomal RNA (rRNA) processing (Giaever et al., 2004; Lum et al., 2004), an observation that was subsequently confirmed in follow-up studies showing that 5-FU blocks rRNA processing by the exosome (Lum et al., 2004).

Homozygous profiling (HOP) is similar to HIP but instead uses complete homozygous deletions to identify genes that confer resistance to a drug. This assay can be used to identify direct targets, as demonstrated by its ability to identify FKBP12 and TOR as targets of rapamycin (Heitman et al., 1991). In many cases, this assay is used to identify genes in pathways that buffer the effects of the compound. The HOP assay is useful in studies where there is no specific protein target or where the target is known and identification of genes that interact with the target is required. An important application of HOP is to identify resistance genes in pathways that may be functionally redundant or have high transcriptional compensation in the cell, and has been particularly powerful for identifying genes that are involved in the DNA damage response (DDR) (Birrell et al., 2001; Chang et al., 2002; Workman et al., 2006).

Examples of the use of HOP to study DDR include a study by Birrell et al. (2001), where the assay was used to identify genes involved in UV radiation sensitivity. The authors were able to identify genes known to be involved in DNA repair pathways, such as nucleotide excision repair, cell-cycle checkpoints, homologous recombination, and postreplication repair. This study led to the identification of three genes (*THR1*, *LSM1*, and *YAF9*) not previously known to be involved in DNA damage repair pathways. Two of these genes have human orthologs associated with cancer. Lee et al. (2005) used the HOP assay to identify genes required for resistance to DNA-damaging agents with diverse mechanisms of action. In this study, 12 compounds that damage DNA were tested to uncover genes involved in distinct DDR modules that are important to repair damage by each compound. The authors found that relative importance of different DDR modules was able to distinguish between compound mechanisms. Specifically, they identified genetic determinants required for resistance against DNA interstrand crosslinking agents. In addition, genes that were previously unlinked to DDR pathways were found in this study.

The recent availability of a genome-wide haploid deletion mutant library in the fission yeast *Schizosaccharomyces pombe* has made chemical genomic screens possible in this distantly related yeast (Kim et al., 2010). Several groups have screened this library against DNA-damaging agents to uncover genes in the *S. pombe* DDR and to assign functions to uncharacterized genes (Deshpande et al., 2009; Pan et al., 2012). *S. pombe* diverged from *S. cerevisiae* up to 1,000 million years ago, and each species shares homologs with metazoans that the other lacks (Forsburg, 2005); therefore, comparative chemical genomic screens in both organisms can be used to better characterize compound mechanism of action and to inform human studies. Kapitzky et al. (2010) developed a cross-species screening platform to study compounds of known and unknown function, including several DNA-damaging agents. The study revealed that cross-species profiling is a more ideal predictor of drug mode of action than single-organism profiling and used their approach to identify an uncharacterized compound, NSC-207895, as a DNA-damaging agent. A recent study by our lab used a similar comparative chemogenomics approach by performing HOP assays in *S. cerevisiae* and *S. pombe* to evaluate the potential of 11 platinum-acridine hybrid compounds as anticancer agents (Cheung-Ong et al., 2012). Four platinum-acridine agents elicited responses from DDR genes; interestingly, their mechanisms of action were found to be distinct from the DNA-crosslinking produced by the classical platinum agent cisplatin. The characterization of novel compounds as DNA-damaging agents is of considerable interest for clinical development.

Systematic Screens to Characterize Genetic Responses to DNA Damage

Yeast strains that contain pairs of gene deletions have been extremely useful for studying genetic interactions, also known as epistasis (Costanzo et al., 2010). Taking this concept a step further, perturbing the double mutants (e.g., with drug application) can uncover changes in genetic interactions or novel functional relationships in response to a compound. St Onge et al. (2007) examined genetic interactions between 26 genes whose single deletion mutants conferred resistance to the DNA-alkylating agent methyl methanesulfonate (MMS) in previous HOP assays (Giaever et al., 2002; Lee et al., 2005). The fitness of 650 double mutant strains was examined in pooled liquid culture, similar to the assays described above, to identify alleviating and aggravating interactions. The authors use the resulting interaction network to predict roles for DDR genes, such as a role for Mph1 helicase in resolving recombination-derived DNA intermediates.

Bandyopadhyay et al. (2010) adapted the drug-treated double mutant approach to a colony-based readout, which they called differential epistatic miniarray profiling, which, similar to the St. Onge et al. study, quantifies genetic interactions between pairs of genes and compares the results of two different treatments. Genetic interactions between 418 yeast genes involved in signaling, transcription, and DNA repair were interrogated by generating 80,000 double mutant strains. These strains were grown in the presence or absence of the MMS on solid media and their colony sizes evaluated. The resulting differential epistatic network was highly enriched for DDR genes. Several genes in DNA repair pathways acted as "hubs" for interactions of many known DDR genes. This observation led to the prediction that hub genes previously unlinked to DDR, such as *SLT1* and *CBF1*, could be involved in DDR or related processes. Indeed, Cbf1 was found to be required for cell-cycle checkpoint activation and induction of the DNA damage-indicating histone modification γH2AX. These two studies highlight the utility of combinatorial deletion profiling to study changes in genetic networks in response to DNA damage. The results of these screens may prove important for studies of cancer genetics, since DDR pathways are frequently mutated in cancer cells.

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Another yeast strain collection that has been useful to study cellular responses to DNA-damaging agents is the genome-wide collection of GFP-tagged proteins. Tkach et al. (2012) introduced DNA damage to this collection by addition of MMS or hydroxyurea (HU) and examined global changes in protein levels and localization. Following compound treatment, the authors identified 356 proteins that changed in abundance and 254 that changed in localization with little overlap; enrichment analysis of these proteins indicated that the biological processes differed between these classes. Within the protein localization class, there was significant overlap in localization changes and destination in HU and MMS. Further analysis revealed that proteins that shared the same localization after compound treatment indicated a common biological function. The authors identified an additional class of DDR proteins, including Cmr1, Hos2, Apj1, and Pph21, that form nuclear foci away from the well-characterized DNA double-strand break foci. This study uncovered previously unknown DNA damage response pathways that allow a better understanding of this important cellular pathway and can allow the identification of genes involved in cancer pathways.

Chemical Screens in Multicellular Organisms

Screening compounds in whole organisms is advantageous compared to single-celled organism and cultured cell screens, because, in addition to providing a greater array of phenotypic readouts, it allows for the evaluation of compound toxicity, tissue specificity, and drug bioavailability. To provide a rapid method for evaluating chemical libraries, these model organisms should be amenable to genetic manipulation, and appropriate screening platforms would be required for large-scale screening of compounds. In vivo screening of anticancer compounds allows the selection of compounds that specifically act against tumor cells without adverse toxicity to the host; this approach can be used to identify new anticancer compounds that address the limitations of current therapeutics.

The fruit fly *Drosophila melanogaster* shares many cancer-relevant pathways with humans and can be used to model tumor growth and to evaluate anticancer compounds (for review, see Gladstone and Su, 2011). Two notable studies use cancer models developed in *D. melanogaster* to examine the effects of anticancer compounds in whole animal screens. Willoughby et al. (2013) developed a platform for high-throughput screening of diverse chemical compounds and applied the screens to identify inhibitors of tumor formation in a *D. melanogaster* Ras-driven cancer model. Evaluation of 2,000 compounds uncovered the glutamine analog acivicin as an inhibitor of tumor growth and identified CTP synthase as a potential target of acivicin. Dar et al. (2012) used a Ret-driven model of multiple endocrine neoplasias and demonstrated that in vivo screening and genetic analysis could identify pathways that allow for increased efficacy and reduced toxicity of known compounds. The information gathered in their study may prove extremely important for the design of novel anticancer compounds and/or the development of combination therapies to specifically target genes in these pathways.

The zebrafish *Danio rerio* is well-suited for whole organism screens, because it is evolutionarily closer to humans than *Drosophila* and growth can be readily monitored due to their transparent embryos and discrete organs. *D. rerio* has become a valuable vertebrate model system for modeling cancers (for review, see Huang et al., 2011). Yeh et al. (2008) employed a transgenic model for acute myelogenous leukemia (AML1-ETO) that mimics cell differentiation defects that occur in human AML. A library of 2,000 compounds was screened against these transgenic zebrafish and a cyclooxygenase 2 inhibitor was found to reverse the downstream effects of the transgene. This discovery could lead to enhanced therapeutic potential in combination with antiproliferative drugs for the treatment of AML. Xiang et al. (2009) used a combinatorial chemistry approach to identify tumor cell growth inhibitors that act via cyclin-dependent kinases (CDKs), which are cell-cycle proteins that have abnormal activity in human tumors. CDK inhibitors were designed, synthesized, and rapidly screened against whole zebrafish embryos. A lead compound with selective inhibition of CDK2 function was discovered that also exhibits efficacy in human cells. The combination of synthetic chemistry and whole organism screening provides a streamlined method for the rapid development of target-specific compounds.

Functional Genomics in Mammalian Cells

Model organism research has demonstrated the importance of employing genomics and chemical genomics to understand biological function and human disease. However, although model organisms are excellent test beds for understanding well-conserved processes, there are processes that can only be studied in genetically related cell types. Indeed, adapting loss-of-function and gain-of-function screens performed in model organisms to mammalian systems has long been a goal, and recently, great strides have been made toward this goal. Performing genomic studies in mammalian systems is more challenging on several fronts: genetic manipulation, while improving dramatically, is still difficult, and these screens are cost and labor intensive.

RNA interference is the process by which noncoding double-stranded RNA (dsRNA) molecules mediate target-specific degradation of messenger RNA (mRNA). Observed in *C. elegans* when antisense RNA molecules were tested for their ability to inhibit gene expression (Fire et al., 1998), Fire and Mello found that dsRNA was more potent in triggering gene silencing than single-stranded RNA; this work was recognized with a Nobel Prize in 2006 (for a review of RNA interference [RNAi], see Hannon, 2002). The ease of genetic manipulation through RNAi has produced a paradigm shift in mammalian molecular biology research by accelerating the development of genome-scale functional studies in human cells. RNAi-based loss-of-function studies now allow one to examine human genes directly in cultured cells, either individually or in pooled assays. Both commercial and academic laboratories have generated a number of large genome-scale short interfering RNA (siRNA), short hairpin RNA (shRNA), and endoribonuclease-prepared siRNA (esiRNA) libraries in plasmids and viral vectors that enable large-format screening of human genes. To illustrate the diversity of these resources, The RNAi Consortium has a library of 90,000 shRNA constructs that target ~18,000 human genes in lentiviral vectors (Moffat et al., 2006), the Netherlands Cancer Institute has a library of 24,000 19-mer shRNAs in retroviral vectors (Bernards et al., 2006), and the Hannon and Elledge labs generated a library of microRNA-adapted shRNAs (shRNA-miRs), which contains 395,830 shRNA-miRs against 57,293 human transcripts (Olson et al., 2006).

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The primary advantages to using RNAi for functional genomics are that RNAi molecules can be easily introduced, either transiently (siRNA and esiRNA) or stably (shRNA and shRNA-miR), into cells and screens can be done in arrayed (well-by-well) or pooled formats. A disadvantage to using RNAi is its potential for off-target effects, which can occur when RNAi sequences bind to nontargeted mRNAs. Another limitation is that the efficacy of mRNA knockdown varies (in unpredictable ways), depending on the cell type and the sequence target of the RNAi molecule.

In an arrayed screen, each RNAi molecule is placed into a single well of a microtiter plate. The advantage to this type of screen is that there are no confounding effects from other infected cells, since only one knockdown or one set of knockdowns/gene are assayed. In addition, this format is suitable for multiple types of readouts, including colorimetric, fluorescence, and luminescence assays and high-content morphological phenotyping. The main disadvantages of array-based screens, especially with the large number of molecules in each of these libraries, are that costly infrastructure is required, the volume of reagents is high, and a minimum level of automation is necessary. For pooled approaches, the library is typically introduced into cells en masse and, based on the statistics of infection, each cell, on average, contains a single RNAi molecule. Following the screen, the individual clones can be identified using barcode microarray or sequence-based readouts (Figure 2). Pooled approaches are more feasible for smaller laboratories, but a disadvantage to pooled screens is that analysis of results requires costly deconvolution through microarrays or next-generation sequencing and sufficient bioinformatic expertise to identify the RNAi molecules that are enriched or depleted in a pool. Our lab recently developed a microarray-based platform that allows the deconvolution of pools of up to 90,000 shRNA molecules (Ketela et al., 2011). The technical challenge of identifying specific molecules in highly complex RNAi libraries can be addressed by dividing genome-scale collections into smaller pools (Luo et al., 2009).

Chemical Genomic Screens in Mammalian Cells

Several RNAi-based genome-scale studies have been used to study the molecular effects of DNA-damaging agents on mammalian cells. In a proof-of-principle chemical screen, Luo et al. (2008) performed a pool-based screen on H82 small-cell lung cancer cells infected with $\sim$45,000 shRNAs to identify the target of the DNA-damaging anticancer agent etoposide. As described above, etoposide is a topoisomerase II poison and exhibits increased toxicity with increased cellular levels of the topoisomerase II protein. This positive selection screen, which used a high dose of etoposide, correctly identified *TOPIIA* as a suppressor gene. Cells containing TOPIIA knockdowns exhibited up to 50-fold enrichment in etoposide treatment compared to untreated cells.

RNAi-based chemical screens (i.e., synthetic lethal screens) have also been used to identify previously unknown members in DNA damage-related pathways. In a study by Smogorzewska et al. (2010), the Fanconi anemia-related protein FAN1 was identified in a screen for gene knockdowns that confer sensitivity to the DNA crosslinking agent mitomycin C (MMC). U2OS cells infected with a pool of 75,000 shRNAs were treated with a low dose of MMC and analyzed for relative abundance of shRNAs compared to an untreated control. Among the 2,173 hairpins that conferred sensitivity to MMC, several targeted known DDR proteins, such as BRCA1 and RAD51. The group focused on FAN1, a previously uncharacterized protein, and found that, in addition to conferring resistance to MMC, the protein interacts with known mismatch repair proteins, localizes to sites of DNA damage, and contains endo- and exonuclease activity, which indicates a role in DNA repair.

In an analogous screen, HeLa cells were infected with the same pool of 75,000 shRNAs and treated with the topoisomerase I poison camptothecin (CPT) (O'Connell et al., 2010). The screen uncovered shRNAs targeting TOP1 as enriched in CPT-treated cells. On the opposite side, 331 hairpins were found to confer sensitivity to CPT treatment. Among the genes with multiple shRNAs that induce a synthetic effect, the group discovered a protein related to yeast Mms22p/Mus7p, MMS22L, which interacts with another hit from the screen, NFKBIL2. An independent study also identified MMS22L and NFKBIL2 (TONSL) in an RNAi-based screen to identify siRNAs that lead to increased 53BP1 subnuclear foci formation in response to ionizing radiation (O'Donnell et al., 2010). Subsequent experiments determined that both these proteins promote homologous recombination repair, increase DNA damage foci when knocked down, and accumulate at sites of replication stress and DNA damage (O'Connell et al., 2010; O'Donnell et al., 2010). These studies highlight the power and potential of chemical genomic screens to identify novel genes in known biological pathways.

Perspective

The availability of fully sequenced genomes has facilitated the development of high-throughput technologies to systematically probe gene function and validate drug targets to better understand drug mechanism of action. This has important applications in the development and evaluation of novel drugs to improve on current therapeutics. In conjunction with improvements in medicinal chemistry, systematic genome-wide screens provide an effective method to evaluate structure-activity relationships, enabling the prioritization of compounds for drug development (Wallace et al., 2011). In addition, the identification of new "druggable" genes that arise from genome-wide compound screens can lead to the design of novel drugs to target these genes (Hopkins and Groom, 2002). Results from genomic screens can be combined with other -omics strategies, such as proteomics and transcriptomics, to enhance drug target discovery efforts.

Genome-wide chemical screens are designed to uncover all genes that are important for compound response. The results may represent genes that are important for resistance mechanisms or those responsible for off-target effects of the drug. Chemical genomic screens can be used to inform the development of combination therapy strategies for currently used drugs. The identification of a specific gene dosage mutant that enhances the function of a known compound can be used to select drugs that mimic the gene dosage effect for combination therapies. Such strategies can be used to address issues of drug resistance and also allow for decreased individual drug dose to circumvent adverse dose-related toxicity. In addition to their role in drug discovery, chemical genomic screens allow the identification of compounds that can be used as chemical probes.

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A goal of ours and several research groups is to enable chemical genomic screens to be performed directly in mammalian systems. This will allow the direct study of genes and pathways that do not have homologs in yeast. Though heterologous systems, in which human genes are introduced to model organisms, have been useful for studying human proteins (Outeiro and Lindquist, 2003), examining a protein's response to drug in its native environment would be preferable. Once a technology is established in a "standard" cell line, genomic screens can be tailored to a specific disease-relevant system. In practice, no one cell line can be expected to model all human diseases; instead, a panel of reference lines for each disease, complemented with patient-derived cells, may be the most effective approach. Potential reference systems include primary tumors, cell lines engineered to model disease states, and patient-derived induced pluripotent stem cells (iPSCs) that model specific diseases. An exciting example of disease model to study drug function was demonstrated in a recent study of human iPSCs generated from schizophrenia patient-derived cells (Brennand et al., 2011). The iPSCs were differentiated into neurons to model the disease, and subsequent screening against several antipsychotic compounds revealed that loxapine was able to reverse several schizophrenia-related phenotypes in the differentiated neurons.

In addition to the application of RNAi to functional genomics in mammalian cells, newer technologies are being developed that enable precise genetic manipulation in human cells and should prove scalable to the genome-wide level. One example is the generation of human gene knockout cells using haploid human cell lines (Carette et al., 2009). Loss-of-function chemical screens using this technology allow the study of null alleles, emulating screens in model organisms. Advances in genetic engineering in mammalian systems include the development of zinc finger nucleases (Carroll, 2011) and transcription activator-like effector nucleases (Christian et al., 2010). These engineered nucleases can be used to target endogenous human genes and enable site-directed insertions, deletions, and DNA editing in human cells.

A comprehensive understanding of a drug's mode of action is crucial for its safe and effective use in the clinic. Chemical genomics has proven to be a valuable tool to understand cellular responses to diverse compounds, including DNA-damaging agents. The studies highlighted in this review emphasize the power of chemical genomic screens in model organisms and mammalian systems to identify molecular targets of DNA-damaging agents, determine the biological pathways through which these compounds act, and further characterize the DNA damage response pathway. The DDR pathways have important implications in cancer development, as defects in a number of DDR genes lead to genomic instability and are associated with cancer predisposition; therefore, identifying genes that are required to respond to DNA-damaging agents will allow a better understanding of cancer biogenesis and highlight potential targets for anticancer drug development.

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