Preface

Each human is genetically distinctive, and responds differently to disease-causing factors as well as to drugs. Mechanisms inside human bodies that control drug responses are complex and multifactorial. Pharmacogenomics arose in response to such recognition of the necessity of personalized medicine, a medicine that deals with the complexity of the human body. The development of pharmacogenomics represents the evolution of biomedicine from treating the general disease itself to treating the malfunction of an individual person, the "root" of diseases. With the change of focus from diseases to humans, pharmacogenomics brings hope for the transformation from disease treatment to disease prevention.

Pharmacogenomics is considered the future of drug therapy. For the drug development industry, pharmacogenomics is useful in identifying drug targets to obtain optimal drug efficacy for certain patient populations. Because of the diversity of patients' biological backgrounds, the same disease may be caused by genetic variations in different people, who will respond differently to the same drug. Such situations require individualized treatment that avoids adverse drug responses and ensures the best possible results.

However, many challenges need to be resolved before pharmacogenomics can be applied in the clinic. These challenges include the identification of biomarker genes and pathways, the understanding of interactions between genes and drugs, and the correlation of genotypes to disease and drug response phenotypes.

In this book, we approach these challenges from three aspects. We first introduce some important cutting-edge technologies that are useful for the development of systems-based pharmacogenomics to solve the complexity; these technologies include bioinformatics, microarray, and association studies. These technologies can help us with the identification of biomarker genes and pathways and in understanding the associations among genes, drugs, and diseases.

These systems-based approaches use bioinformatics methods for studies in pharmacogenomics and systems biology to manage, organize, and understand the overwhelming information. Integrated methodologies and procedures for applying bioinformatics analysis in pharmacogenomics are presented in this book, as bioinformatics has become indispensable for almost all biopharmaceutical studies today. Pharmacogenomics-related resources, including databases and tools, are collected and provided.

Microarrays and biochips are powerful technologies for high-throughput (HTP) analysis that may enable systematic understanding of genomics and proteomics as well as large drug response data sets. The applications of microarrays in pharmacogenomics, genotyping, and clinical diagnosis, as well as the evolution and development history of the technology, are introduced in this book. Different techniques, platforms, and tests are also discussed.

Association study is a useful method in pharmacogenomics for investigating how individuals with unique genetic variants respond to a drug treatment. Confounding caused by population structure and admixture can contribute to the lack of replication of association study results. Methods for detecting and adjusting confounding are explained, as are their advantages and disadvantages.

The second aspect of this volume includes approaches to studying gene–drug interactions, that is, how drugs act and how they are processed in the human body, including drug absorption, distribution, metabolism, and excretion. Biomarkers and molecules such as ion channels, membrane transporters, receptors, and enzymes are playing increasingly essential roles in drug design and pharmacogenomics studies. These biomarkers provide critical links between drug discovery and diagnostics efforts. Updated introductions and detailed methods about studies in these molecules are provided in this book.

For example, membrane transporters are profoundly involved in drug disposition through transporting substrate drugs between organs and tissues. Investigations of genetic variations, genotyping methods, and substrate identification of membrane transporters are helpful for drug design and development. Different methods for assessing functional significance of transporter polymorphisms in vitro and in vivo as well as the application of transporter genetics in clinical pharmacology are described. Clinical significance of pharmacogenomics studies in drug-metabolizing enzymes and drug transporters for certain treatments, such as chemotherapy, is discussed in detail.

Studies of G protein-coupled receptors (GPCRs) may provide insight into disease pathways, such as the involvement of the regulator of G protein signaling (RGS) protein polymorphisms in hypertension. Pharmacogenomics of GPCR studies the involvement of genetic variations in structural and functional roles, such as GPCR activation and inactivation, their relationships with diseases, and their potential uses in defining optimized novel drug targets. These investigations can be useful for refining drug discovery as GPCR disorders are associated with a wide variety of human diseases, including retinal diseases, thyroid diseases, obesity, diabetes, asthma, cardiovascular diseases, cancer, and infectious diseases.

The third aspect composes a large part of this book: a focus on how pharmacogenomics can be used in therapeutics of diseases. These diseases include cardiovascular diseases, cancer, neurological diseases, gastrointestinal disorders, autoimmune diseases, and infectious diseases. Comprehensive information for each disease system is discussed, including biomarkers involved in the disease and the associations among genes, drugs, diseases, drug response phenotypes, and the environment.

For example, epigenetics and environmental factors may play important roles in major psychiatric disorders. Detailed methods for studying these factors are given to provide a prototype model system for better diagnosis and management of

mental diseases. Asthma is another disease caused by interactions among multiple causes, including demographic, social, environmental, and genetic factors. The most common biological pathways targeted by asthma therapy and the genetic contributions to varied therapeutic responses are described.

Drug treatment in Alzheimer's disease (AD) accounts for more than 10% of direct costs, while fewer than 20% of AD patients are fair responders to conventional drugs. Pioneering pharmacogenomics studies have shown that the therapeutic response in AD is genotype specific as pharmacogenomics factors account for more than 60% of drug variability in drug disposition. This book provides a comprehensive and detailed discussion of the pharmacogenomics of AD, from functional genomics to therapeutic strategies. The integration of these pharmacogenomics protocols with AD drug discovery and clinical practice can help promote therapeutics optimization and develop cost-effective pharmaceuticals to improve both drug efficacy and safety.

For cardiovascular diseases, methods for choosing candidate genes and single-nucleotide polymorphisms (SNPs) and the association with functional studies are discussed. These mechanistic studies are particularly important when it comes to pharmacogenomics associations. These studies provide significant and clinically relevant insights into the variable drug responses in cardiovascular disease management.

In gastroenterology and hepatology, genetic variations involved in drug metabolism or disease pathophysiology have been found to have an impact on drug responses. Discussions in this book focus on clinical pharmacogenomics of inflammatory bowel disease, *Helicobacter pylori* infections, gastroesophageal reflux disease, irritable bowel syndrome, liver transplantation, and colon cancer.

For rheumatoid arthritis, the pharmacogenomics of three major disease-modifying antirheumatic drugs (methotrexate, azathioprine, and sulfasalazine) and one class of biologic antirheumatic drugs (the tumor necrosis factor antagonists) are discussed in detail.

Cancer pharmacogenomics includes studies on biomarkers such as thiopurine methyltransferase (TPMT) and epidermal growth factor receptor (EGFR). Research methods such as germline and tumor DNA studies, polymorphism selection, and biomarker screening as well as genotyping systems are described.

Using array technology in pharmacogenomics, efficacy and systemic toxicity can be evaluated for the improvement of the design and development of preclinical vaccines. Methods of applying pharmacogenomics in the evaluation of efficacy and adverse events during clinical development of vaccines are also discussed.

By covering topics from individual molecules to systemic diseases, from fundamental concepts to advanced technologies, this book intends to provide a practical, state-of-the-art, and integrative view of the application of pharmacogenomics in drug discovery and development. I would like to thank all of the authors for their contributions to this exciting new field. I also thank the series editor, Dr. John Walker, for his help with the editing.

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Chapter 2
Applications of Microarrays and Biochips in Pharmacogenomics

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Summary The complete sequence of the human genome and subsequent intensive searches for polymorphic variations are providing the prerequisite markers necessary to facilitate elucidation of the genetic variability in drug responses. Improvements in the sensitivity and precision of DNA microarrays permit a detailed and accurate scrutiny of the human genome. These advances have the potential to significantly improve health care management by improving disease diagnosis and targeting molecular therapy. Pharmacogenetic approaches, in limited use today, will become an integral part of therapeutic monitoring and health management, permitting patient stratification in advance of treatments, with the potential to eliminate adverse drug reactions. In this chapter, the current state of biochip technology is discussed, and recent applications in the arena of clinic diagnostics are explored.

Keywords AmpliChip; biochips; microarrays; P450; pharmacogenetics.

2.1 Introduction

The sequencing of the human genome has been widely touted as a critical scientific milestone that will revolutionize the process of drug discovery. The continuing analysis of the human genetic code will provide the scientific framework on which

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it may be possible to identify novel potential drug targets, the common genetic factors that can affect drug metabolism and toxicity, and the genetic factors that contribute to the wide variability in pharmacological treatment responses routinely observed in clinical settings. The ever-increasing utilization of genetic techniques, including microarray technologies, has provided a means by which geneticists, biologists, and pharmacologists have begun to bridge the gap between gene sequence and function. These newer approaches are currently under integration into multiple aspects of the drug discovery process. The use of genetic polymorphism analysis has been applied to target validation, pharmacokinetics and toxicology, and clinical pharmacogenomics, while microarray technologies have been utilized in target validation, *in vitro* pharmacology, and toxicology ***(1)***.

A DNA microarray (also referred to as gene or genome chip, DNA chip, or biochip) is a collection of microscopic DNA features attached to a solid support, commonly glass, plastic, or silicon. The array features or "spots" contain DNA probes that are used to interrogate individual genes or polymorphisms. Most arrays in use today contain hundreds to thousands of probes. The value of this technology is that it permits highly parallel measurements. In the case of gene expression profiling, the massive number of data points obtained from a single experiment provides insight into the state of a transcriptome in, for example, healthy and diseased cells or cells before and after exposure to a therapeutic treatment. The knowledge obtained from such comparisons is incredibly compelling as it permits the identification of gene families and pathways pertinent to the malady or drug treatment in addition to those that remain unaffected. Similar expression profiles may infer that genes are coregulated, enabling the formulation of hypotheses about genes with hitherto unknown functions by comparison of their expression patterns to well-characterized genes ***(2)***.

The applicability of microarrays in genomics research has expanded with the evolution and maturation of the technology. Biochips have found utility in exon-based gene expression analyses, genotyping and resequencing applications, comparative genomic hybridization studies, and genomewide (epigenetic) localization ***(3)***. Biochips are widely applied to improve the processes of disease diagnosis, pharmacogenomics, and toxicogenomics ***(4–7)***. In this chapter, the evolution of biochip platforms is reviewed; I compare and contrast platforms currently in use and discuss biochips in the context of pharmacogenetic testing.

2.2 Pharmacogenetic Testing and Health Care

Pharmacogenetics is the discipline that studies the relationship between a patient's inherited genetic makeup and that patient's response to pharmaceutical drugs. Pharmacogenetic testing aims at determining the underlying genotypic and phenotypic differences in the pharmacodynamics and pharmacokinetics of drug metabolism. Whereas pharmacogenetics refers to genetic differences (variation) in drug metabolism and response, *pharmacogenomics* refers to study of the multiplicity of

genes that ultimately determine drug behavior. Pharmacogenomics is in essence the whole-genome application of pharmacogenetics, correlating gene expression or single-nucleotide polymorphisms (SNPs) with drug efficacy and toxicity. Genetic variability in drug response occurs as a result of molecular alterations in the enzymes involved in the metabolism of a particular drug in addition to the drug receptors and transport proteins ***(8)***.

A recent advance and fundamental shift in health care has been the emergence of personalized medicine. Drug–drug interactions (DDIs) can have serious consequences, such as adverse drug reactions (ADRs), and extreme outcomes, including death. DDIs have become a serious issue, particularly in the care of elderly patients, who are often prescribed a wide variety of medications ***(9)***. ADRs are presently the fourth leading cause of death in the United States, resulting in 106,000 deaths per year, and the fifth leading cause of illness, resulting in 2.2 million hospitalizations annually. At present, approx. 28% of adults and 17% of children hospitalized have drug-related ADRs. The economics of drug-related morbidity and mortality has become a pressing issue, with current costs estimated at $177 billion annually ***(10)***.

Pharmacogenetic approaches, in limited use today, will in the near future become an integral part of the therapeutic monitoring and health management of patients. A major advantage of pharmacogenetic testing over classical therapeutic drug monitoring (TDM) approaches is that patient genotyping and stratification can be carried out in advance of drug treatments, thereby eliminating or reducing adverse effects. Testing can generally be performed in a noninvasive manner using DNA obtained from saliva, hair root, or buccal swab samples. Another benefit over traditional methods is that patient compliance with a particular treatment regimen is not required. In addition, the results remain constant over the lifetime of an individual, regardless of disease or aging. Finally, a major advantage of pharmacogenetic testing is that it can provide predictive value for many drugs rather than a single drug ***(8)***.

2.3 Important Pharmacogenetic Targets

The most relevant pharmacogenetic targets as defined by the American Association of Clinical Chemists (AACC) include the Cytochrome P450 enzymes CYP2D6, CYP2C9, CYP2C19, CYP3A5, CYP2B6 and thiopurine s-methyltransferase (TPMT), N-acetyltransferase 2 (NAT2), UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), multi-drug-resistance (MDR1) gene and methylenetetrahydrofolate reductase (MTHFR). Drug metabolism occurs largely in the liver and involves cytochrome P450 (CYP450), a large family of oxidative enzymes. The name derives from “pigment at 450 nm” as the majority of family members possess red coloration owing to the presence of heme at the active site. Although CYP450 plays an important role in the synthesis and breakdown of hormones, cholesterol synthesis, and vitamin D metabolism, from a health care perspective its role in drug metabolism is its most pertinent. Most common variations in drug metabolism

between individuals can be explained by polymorphisms in the *cypP450* genes. One of the best characterized of the CYPP450 enzymes, CYP2D6, is responsible for metabolizing the majority of pharmaceuticals currently in use. These include an extensive range of therapeutic agents encompassing β-blockers, antidepressants, antipsychotics, and opioids. A poor metabolizer (PM) phenotype has been observed among 7–10% of the Caucasian population, with many suffering toxicity from normally prescribed doses. This is explained by adverse reaction to drugs prescribed in standard doses or undesirable DDIs when using multiple-drug therapeutics.

Warfarin (Coumadin) inhibits the synthesis of clotting factors, thus preventing blood clot formation. Although it remains the most frequently prescribed oral anticoagulant, it can cause severe bleeding that can be life-threatening and cause death. Successful management of warfarin therapy is problematical owing to the wide variation in drug response among patients. Variation in the vitamin K epoxide reductase complex 1 (VKORC1) gene affects the response to warfarin ***(11)***. Pharmacogenetic analysis of a patient's *CYP2C9* or *VKORC1* can provide information that allows fine-tuning of the appropriate warfarin dosage. Cytochrome P4502C19 metabolizes 15% of all prescribed drugs and is involved in the metabolism and clearance of antidepressants (tricyclic antidepressants [TCAs] and selective serotonin reuptake inhibitors [SSRIs]), anticonvulsants, anxiolytics, and benzodiazepines ***(12–14)***. For 2C19, two phenotypes with variable metabolic activity have been defined, the extensive metabolizer (EM) and poor metabolizer (PM). The PM phenotype is associated with low enzyme activity. East Asians are most likely to exhibit the PM phenotype, with 2C19 PM rates observed in up to 25%. CYP4503A4/3A5 is the most abundant CYP450 isoenzyme in humans and is responsible for the metabolism of the widest range of drugs. It is involved in the metabolism and clearance of calcium channel blockers, benzodiazepines, human immunodeficiency virus (HIV) protease inhibitors, HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors, and antithrombolytics.

Thiopurine s-methyltransferase (TPMT) catalyzes the S-methylation or inactivation of the thiopurine drugs mercaptopurine, azathioprine, and thioguanine, which are commonly used to treat leukemia, rheumatic diseases, and inflammatory bowel disease. *TMPT* testing serves to detect patients at risk of developing side effects if treated with thiopurine drugs ***(12)***. *N*-Acetyltransferase 2 (NAT2) is of clinical importance as rapid or slow acetylation of therapeutic and carcinogenic agents is explained by variability at the *NAT2* locus. Interethnic variations in distribution of the acetylation phenotype are significant.

UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), is a hepatic enzyme associated with the colorectal and small lung cancers. UGT1A1 metabolizes irinotecan, an antineoplastic agent utilized for the treatment of colorectal cancer. Pharmacogenetic testing for *UGT1A1* will help the optimization of therapeutic approaches with antineoplastic agents that inherently have a low therapeutic index and will spare patients from excessive toxicity resulting from therapy with irinotecan.

P-Glycoprotein (P-gp), a member of the adenosine triphosphate (ATP)-binding cassette family of membrane transporters, is encoded by the human multidrug-resistance (*MDR1*, *ABCB1*) gene ***(15)***. This integral membrane protein serves as an

energy-dependent drug efflux pump and reduces the intracellular concentrations of a wide range of drugs and xenobiotics. The overexpression of MDR1 is associated with resistance to doxorubicin, taxanes, and vinca alkaloids, which are used to treat cancer. Resistance to chemotherapy has become a major obstacle in anticancer treatment. Methylenetetrahydrofolate reductase (MTHFR) is a cytoplasmic enzyme that plays a role in the conversion of homocysteine (a potentially toxic amino acid) to methionine. A common 677TT genotype predisposes individuals to mild hyperhomocysteinemia (high blood homocysteine levels), which can lead to neural tube defects in offspring, arterial and venous thrombosis, and cardiovascular disease.

Currently, the methods employed for genetic testing are labor intensive and intricate and demand the concurrent analysis of multiple nucleic acid markers. Microarray technology is undeniably the most practical approach to multiplex and analyze biomolecular markers.

2.4 Evolution and Development of Microarrays

The origin of the microarray or biochip can be traced to a seminal publication by Edwin Southern over 30 years ago. Southern described a method by which DNA could be attached to a solid support following electrophoresis and interrogated for sequences of interest by hybridization with a complementary DNA sequence ***(16)***. The complementary DNA sequence, termed a *probe*, was labeled with either a radioactive or a fluorescent marker and hybridized to the DNA target sample, which was immobilized on a solid support, such as a nitrocellulose filter membrane.

The biochips widely in use today owe their existence to innovations in miniaturization, DNA synthesis and attachment chemistries, and improvements in image acquisition. Key pioneers in the early innovation and development of this technology were Hyseq (Sunnyvale, CA); Affymetrix (Affymax) (Santa Clara, CA); Oxford Gene Technologies (Oxford, UK); and Stanford University (Palo Alto, CA). Hyseq exploited oligonucleotide arrays to permit sequencing of target nucleic acid sequences. The complementary oligonucleotide probe sequences overlapped, permitting the discrimination of perfect match DNA hybrids from hybrids that contained a single-nucleotide mismatch ***(17)***. Affymetrix utilized very large scale immobilized polymer synthesis (VLSIPSTM) substrate technologies for the synthesis of both peptides and oligonucleotides on solid supports. They successfully applied this technology to DNA sequencing, DNA fingerprinting, chromosomal mapping, and specific interaction screening ***(18)***. Spotted microarrays, yet another widely utilized application of this technology, were pioneered at Stanford University by Patrick Brown and colleagues. These arrays are fabricated using a capillary dispenser, which deposits DNA at specific array positions. Spotted microarray production is highly automated, utilizing either capillary pin-based or ink-jet microdispensing liquid-handling systems ***(19,20)***.

The major commercial microarray platforms in use today, over ten years after their first description, include those from Affymetrix, Illumina, Agilent, and

Applied Biosystems. A detailed comparison and contrast of the salient features of each of these platforms has been described previously ***(21,22)***. The Affymetrix GeneChip™ has been the most extensively used owing to its extensive genome coverage, its ease of use, and its high level of reproducibility. It is comprised of short single-stranded oligonucleotides and is fabricated via a combination of photolithography and solid-phase DNA synthesis. Illumina (San Diego, Ca) has established a bead-based technology that was utilized initially for SNP genotyping and subsequently for gene expression profiling. These arrays are comprised of thousands of tiny etched wells, into which thousands to hundreds of thousands of 3-μm beads randomly self-assemble. Then, 50-mer gene-specific probes linked with "address or zip code" sequences are immobilized on the bead surface and are used to facilitate a decoding process, which maps a specific bead type containing a particular sequence to a given location on the array.

Applied Biosystems Expression Array System (Foster City, CA) has devised a chemiluminescence-based microarray platform utilizing 60-mer oligonucleotides which are validated offline by mass spectrometry and are subsequently printed onto a derivatized nylon substrate. Agilent Technologies (Palo Alto, CA) also utilizes 60-mers, which are synthesized in situ by ink-jet printing using phosphoramidite chemistry.

2.5 Microarrays and Genotyping

Single-nucleotide polymorphisms are highly abundant, with over 10 million present in the human genome, and they serve as valuable markers of genomewide variation. A chromosome region may contain many SNPs, but just a few "tag" SNPs are required to provide information on the pattern of genetic variation. The high costs associated with most SNP detection strategies have until recently made genomewide approaches impractical.

Illumina bead-based technology has been applied to both SNP genotyping and gene expression profiling applications and utilizes two distinct substrates, the Sentrix LD BeadChip and the Sentrix Array Matrix (which multiplex up to 8 and 96 samples, respectively). Genomewide genotyping of defined sets of hundreds of thousands of SNPs can be performed using one of two array types, the Infinium I 109K SNP arrays or the Infinium II 317K SNP arrays. A whole-genome amplification step is initially employed to enrich the target DNA up to 1000-fold. Once amplified, the DNA is subsequently fragmented and mobilized by hybridization to SNP-specific primers present on the array. In the case of the Infinium I assay, which utilizes an allele-specific primer extension approach, the DNA is hybridized to allele-specific primers that are extended with multiple labeled bases only if a perfect match exists between the target and SNP-specific probe ***(23)***. The Infinium II assay differs in that it is based on single-base extension (SBE). An oligonucleotide primer is hybridized adjacent to the SNP site and is extended with a single labeled dideoxy-nucleotide terminator corresponding to the minor or major allele. Genotyping calls can then be made based on the dye-labeled terminator that is incorporated ***(24)***.

2.6 Microarrays and Clinical Diagnostics

Microarrays are today applied in the clinical diagnostics and genotyping arenas. Their successful utilization and survival in the clinic will depend on the ability of the technology to meet the rigorous requirements applied to human diagnostics in a cost-effective manner.

2.6.1 Roche Diagnostics AmpliChip

The first pharmacogenetic microarray-based test approved for clinical use is the AmpliChip CYP450 from Roche Diagnostics (Basel), which measures genetic variation, both deletions and duplications, for the *CYP2D6* and *CYP2C19* genes. The AmpliChip is a marriage of expertise in polymerase chain reaction (PCR; Roche) and microarray (Affymetrix) technologies. The AmpliChip has been approved for *in vitro* diagnostic use in the United States and Europe. The test determines the associated predictive metabolizer phenotype (poor, intermediate, extensive, or ultra) and can aid physicians in individualizing patient treatment and dosing for drugs metabolized through these *P450* genes. It detects a total of 27 polymorphisms and mutations for the *2D6* gene and 3 polymorphisms for the *2C19* gene.

Once patient genomic DNA has been extracted, the test involves a series of five steps, and the analysis time from start to finish is 8 h. A minimum of 25 ng of input genomic DNA is required for the assay, and the preferred tissue source is blood, although buccal swab-derived DNA would also suffice. First, PCR amplification is carried out to amplify the genes of interest using gene-specific primers. This is followed by fragmentation and biotin labeling of the amplicons at their 3′ termini with terminal transferase (TdT). The biotin-labeled amplicon is subsequently hybridized to the AmpliChip DNA microarray. Following washing and staining via a strepavidin–phycoerythrin conjugate, the chip is scanned on an Affymetrix GeneChip Scanner 3000Dx, the data feature is extracted and analyzed, and genotyping calls are made.

2.6.2 Autogenomics BioFilm Microarrays

The Infiniti Analyzer, an automated, continuous-flow microarray platform for clinical applications has been developed by Autogenomics (Carlsbad, CA) ***(25)***. The underlying component of the Autogenomics technology is the BioFilm™, which consists of multiple layers of porous hydrogel matrices 8- to 10-μm thick on a polyester solid base. This provides an aqueous microenvironment that is highly compatible with biological materials. The BioFilm microarray is configured with 15 × 16 arrays (240 spots) per chip, suitable for current diagnostic applications, and permits analyses of both nucleic acid and proteins ***(26)***. It can be tailored to clinical genetic testing for custom polymorphisms of interest.

The analyzer integrates all the discrete processes of sample handling, reagent management, hybridization, and detection. A confocal microscope has been integrated into the analyzer; it has two lasers (red and green). In addition, a thermal stringency station and a thermal cycler for denaturing nucleic acids for primer extension studies or hybridization reactions in solution have been incorporated. A CYP2D6 assay has been designed to detect the most prevalent and informative *CYP2D6* allele variants ***(25)***. The target regions of the *CYP2D6* gene are amplified via a multiplex PCR reaction with specific primer and reaction conditions that can discriminate *CYP2D6* from its pseudogenes. The PCR multiplex reaction is followed by the incorporation of fluorescently labeled nucleotides via primer extension and hybridization of the labeled targets to immobilized oligonuleotides on the BioFilm. Other pharmacogenetic specific tests that can be carried out on this platform include, CYP2C9, CYP2C19, TPMT, CYP3A4/5, and NAT2.

2.6.3 Nanogen NanoChip™

An interesting development has been that of electronic chip technology. Nanogen (San Diego) developed the NanoChip™, which exploits the charged nature of biological molecules. Electronic charges can rapidly shift molecules from one location to another and concentrate them at defined sites on an array. The concentration of biological materials with electronics enables rapid hybridization reactions; instead of the 12 to 16 h traditionally required for passive hybridization, electronic hybridization reactions can be performed in 2 min. When a test site on the NanoChip is charged, a nucleic acid target rapidly moves to that site. Other sites, which are not charged, do not attract the target. Each site or feature can be individually charged electronically via platinum wires and can contain an individual assay or experiment. Electronic hybridization and stringency can be carried out with single-base resolution.

Nanogen has developed pharmacogenetics research reagents for the analysis of *CYP2C9* and *VKORC1*, mutations of which have relevance to warfarin dose optimization. The reagents can be used to rapidly determine genotypes for up to 78 patient samples. In November 2007, Nanogen announced it would be closing its microarray business and repositioning of the company with a focus on real-time PCR and point-of-care testing units.

2.7 Microarray Technology Limitations and Challenges

The commercial microarray platforms in use today have established efficiencies regarding signal dynamic range, the ability to discriminate related messenger RNA (mRNA) species, the reproducibility of the data (raw data, fold change and expression levels). However, technological and standardization limitations exist with

biochip technologies. Expression microarrays facilitate the analysis of the relative levels of mRNA species in one tissue sample compared to another. Although a measure of transcript abundance is achieved, biochips do not provide absolute quantification of the specific mRNA. Microarrays are further limited by the certainty that the data obtained merely indicate whether a given mRNA is above the system's threshold level of detection. If the signal is significantly above the background intensity, then one can say with confidence that the transcript is expressed in that tissue. However, the absence of signal does not indicate the lack of expression. It merely indicates that it is below the detection capability of the system, and there is still a probability that the mRNA is expressed, albeit at basal levels, and this low-level expression may be of biological relevance.

Expression analysis using DNA microarrays analyzes only the transcriptome; it should be mentioned that mRNA abundance in a cell often correlates poorly with the amount of protein synthesized ***(27)***. Important regulation takes place at the levels of translation and enzymatic activities. The only effect of a signal transduction pathway that is observed in a gene expression experiment is that at the endpoint of a given pathway. DNA microarrays currently have little value in determining post-translational modifications, which influence the diversity, affinity, function, cellular abundance, and transport of proteins.

2.8 Conclusion

Currently, the methods employed for genetic testing are both labor intensive and highly complex and require the simultaneous analysis of multiple nucleic acid markers. Microarray technology is without doubt the most practical approach to multiplex and analyze biomolecular markers. Although widely used in the research setting, adaptation of microarray technology to the clinical environment has been slow.

The success of microarrays in the clinical laboratory will depend on their ability to adapt to the rigorous environment of routine usage while providing high-quality, reproducible, and robust results. The clinical environment stretches the limits of this technology as it measures performance criteria in a different manner compared to the research environment. One difference from an economic standpoint is that the cost per reportable result is more important than the cost per data point. Other key factors are the requirements for automation from sample processing to end result, precision, accuracy of results, and the ability to process large volumes of tests under strict regulatory guidelines and compliances.

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2 Murine L1210 and P388 Leukemias

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CONTENTS

1. INTRODUCTION

Mouse leukemia models were a central component of the initial drug discovery programs employed by the Division of Cancer Treatment (DCT) of the National Cancer Institute (NCI) during the early 1960s and 1970s. The L1210 and P388 leukemias, developed in 1948 *(1)* and 1955 *(2)*, respectively, played a major role in both screening and detailed evaluations of candidate anticancer agents. Today, 40 yr later, these models are still used to evaluate anticancer activity, although at a greatly reduced level, and to study mechanisms of drug resistance. This chapter reviews their past contributions, updates their present role in the evaluation of anticancer drugs, and summarizes data for the drug sensitivity of these two leukemias and various drug-resistant P388 sublines to clinically useful drugs.

2. ROLE IN DRUG SCREENING

Spontaneous tumors in animals were first used as models for screening potential anticancer agents. In fact, these types of studies occurred even prior to the beginning of the twentieth century *(3)*, and provided the basis for modern drug-screening programs. However, large-scale screening and the ability to conduct detailed drug-evaluation studies with anticancer agents increased greatly in the 1920s through the development of inbred strains of mice that allowed investigators to propagate tumor lines by serial transplantation in vivo *(4)*.

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The US Congress became interested in cancer research when it was recognized in the 1940s that systemic cancer could be influenced by drug treatment. This was demonstrated at Memorial Sloan-Kettering, one of the first of several institutions in the United States and Europe to begin drug-screening programs. In this program, the mouse sarcoma SA-180 was used as its screening model. However, as drugs exhibited anticancer activity and the supply of new candidate agents exceeded the screening capacity of that program, the need for additional drug development capabilities became apparent. With this impetus, Congress directed NCI to implement a national drug development program, which went into effect in 1955 as the Cancer Chemotherapy National Service Center (CCNSC).

Initially, the CCNSC primary screening program consisted of L1210 leukemia, SA-180, and mammary adenocarcinoma 755 *(5)*. Over the years, the composition of the primary screen changed several times—i.e., from the original three tumors to L1210 and two arbitrarily selected tumors; to L1210 and Walker 256 carcinosarcoma; to L1210 and P388 leukemia; and finally, to L1210, P388, and either B16 melanoma or Lewis lung carcinoma (LLC) *(6)*. Several other models were also used during this period for special, detailed drug evaluation.

The primary screening program underwent a major change in 1976, when DCT incorporated the use of three human tumor xenograft models. The new screen now consisted of a panel of colon, breast, and lung tumors, both murine and human. However, all drugs intended this screen were still initially evaluated for activity against the sensitive P388 leukemia model *(7)*. During this period, the small number of drugs discovered with marked antitumor activity against human solid tumors led to a radical change in the screening program that had used murine leukemia models as the primary screen. In the mid-1980s, NCI developed a new primary screen based on the use of established human tumor-cell lines in vitro *(8)*. The new and old screen programs were to be conducted in parallel to permit a comparison; however, in early 1987, budget cuts at NCI forced an end to large-scale P388 screening *(9)*.

3. CHARACTERISTICS

Both L1210 and P388 leukemias were chemically induced in a DBA/2 mouse by painting the skin with methylcholanthrene *(1,2)*. Propagation of the leukemia lines occurs in the host of origin by intraperitoneal (ip) implantation of diluted ascitic fluid containing either 10^5 (L1210) or 10^6 (P388) cells per animal. Testing is generally conducted in a hybrid of DBA/2 (e.g., $CD2F_1$ or $B6D2F_1$), because the hybrids are somewhat heartier. However, DBA/2 mice may be used for special studies, and should be used for serial in vivo propagation of the leukemias. Frequently used implant sites are ip, subcutaneous (sc), intravenous (iv), or intracerebral (ic). For L1210 leukemia with an implant of 10^5 cells, the median days of death and the tumor doubling times for these implant sites are 8.8, 9.9, 6.4, and 7.0 d and 0.34, 0.46, 0.45, and 0.37 d, respectively. For P388 leukemia with an implant of 10^6 cells, the median days of death and the tumor doubling times for these implant sites are 10.3, 13.0, 8.0, and 8.0 d and 0.44, 0.52, 0.68, and 0.63 d, respectively.

Skipper and colleagues at the Southern Research Institute determined the rate of distribution and proliferation of L1210 leukemia cells using bioassays of untreated mice after ip, iv, and ic inoculation *(10)*. Following ip inoculation, most of the L1210 cells were found in the ascites fluid of the peritoneal cavity. Using the median day of death

as the evaluation time-point, the most commonly infiltrated tissues were the bone marrow, liver, and spleen. Following iv inoculation, the majority of L1210 cells appeared in the bone marrow. On the median day of death from the iv implant, the most infiltrated tissues were also the bone marrow, liver, and spleen. After ic inoculation, most of the L1210 cells remained in the brain (for 3–5 d). On the median day of death from the ic implant, the spleen was heavily infiltrated (the extent of the leukemia in other tissues was not reported).

Southern Research was one of the first institutions to become involved in the CCNSC screening program, and was heavily involved in designing protocols for the program. One aspect essential to the operation of a screening program is the development of appropriate parameters for measuring antitumor activity. At Southern Research, antitumor activity for leukemia studies is assessed on the basis of percent median increase in lifespan (% ILS), net $\log_{10}$ cell-kill, and long-term survivors. Calculations of net $\log_{10}$ cell-kill are made from the tumor-cell population doubling time that is determined from an internal tumor titration consisting of implants from serial 10-fold dilutions *(11)*. Long-term survivors are excluded from calculations of % ILS and tumor-cell-kill. To assess tumor-cell-kill at the end of treatment, the survival time difference between treated and control groups is adjusted to account for regrowth of tumor-cell populations that may occur between individual treatments *(12)*. The net $\log_{10}$ cell-kill is calculated as follows:

$$\text{Net } \log_{10} \text{ cell-kill} = \frac{\text{(T-C)} - \text{(duration of treatment in days)}}{3.32 \times T_d}$$

where (T-C) is the difference in the median day of death between the treated (T) and the control (C) groups, 3.32 is the number of doublings required for a population to increase 1-$\log_{10}$ unit, and T_d is the mean tumor doubling time (days) calculated from a log-linear least-squares fit of the implant sizes and the median days of death of the titration groups.

4. SENSITIVITY TO CLINICAL AGENTS

The majority of clinically useful compounds in current use was first detected in the murine leukemia models. The sensitivities of L1210 and P388 leukemias (ip implantation) to most of these agents (ip administration) are shown in Figs. 1 and 2 and Figs. 3 and 4, respectively. Overall, P388 leukemia is somewhat more sensitive than L1210 leukemia. For alkylating agents, the sensitivities are similar. Notable exceptions are chlorambucil, mitomycin C, and carboplatin, for which P388 is markedly more sensitive. For antimetabolites, the sensitivities are also similar. Exceptions are floxuridine (P388 being markedly more sensitive) and hydroxyurea (L1210 being markedly more sensitive). For DNA-binding agents, P388 leukemia is clearly more sensitive (e.g., actinomycin D, mithramycin, daunorubicin, teniposide, doxorubicin, and amsacrine). For tubulin-binding agents, P388 leukemia is again clearly more sensitive. The vinca alkaloids are active against P388 leukemia, but ineffective against L1210 leukemia.

Although most of the sensitivity data are for ip-implanted leukemia and ip-administered drugs, valuable information can be obtained from separating the implant site and the route of administration. Table 1 shows the activity of melphalan (ip administration) against both L1210 and P388 leukemias implanted through ip, iv, and ic meth-

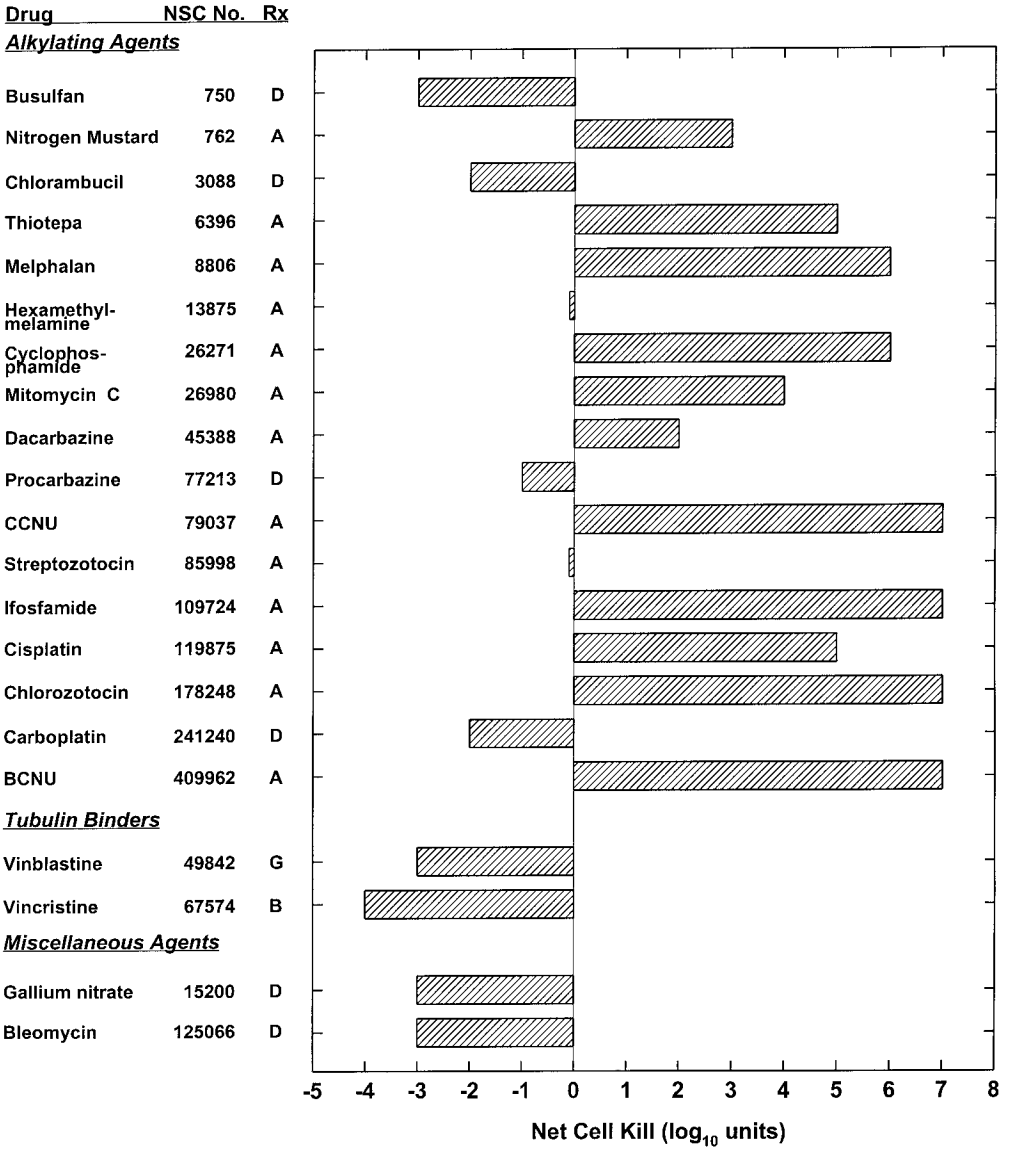

Fig. 1. Sensitivity of ip-implanted L1210 leukemia to clinically useful alkylating agents, tubulin binders, and other miscellaneous agents. L1210 leukemia (10^5 cells except for hexamethylmelamine, which used 10^6 cells) was ip-implanted on d 0. Beginning on d 1, the agents were ip-administered using the indicated schedules. Treatment schedule (R_x): A = d 1; B = d 1, 5, 9; C = d 1–5; D = d 1–9; E = d 1, 4, 7, 10; F = q3h × 8, d 1, 5, 9; G = d 1–15.

ods. The use of ip melphalan is very effective against both ip-implanted leukemias. The activity is reduced to less than one-half when changed to an iv implant site. The activity is further reduced with change to an ic implant site; however, melphalan can cross the blood-brain barrier to some extent. This principle is illustrated more fully with the data in Figs. 5 (L1210) and 6 (P388) for the leukemias with ic implantation, and various clinically useful agents with ip administration. Thiotepa, CCNU, BCNU, and ara-

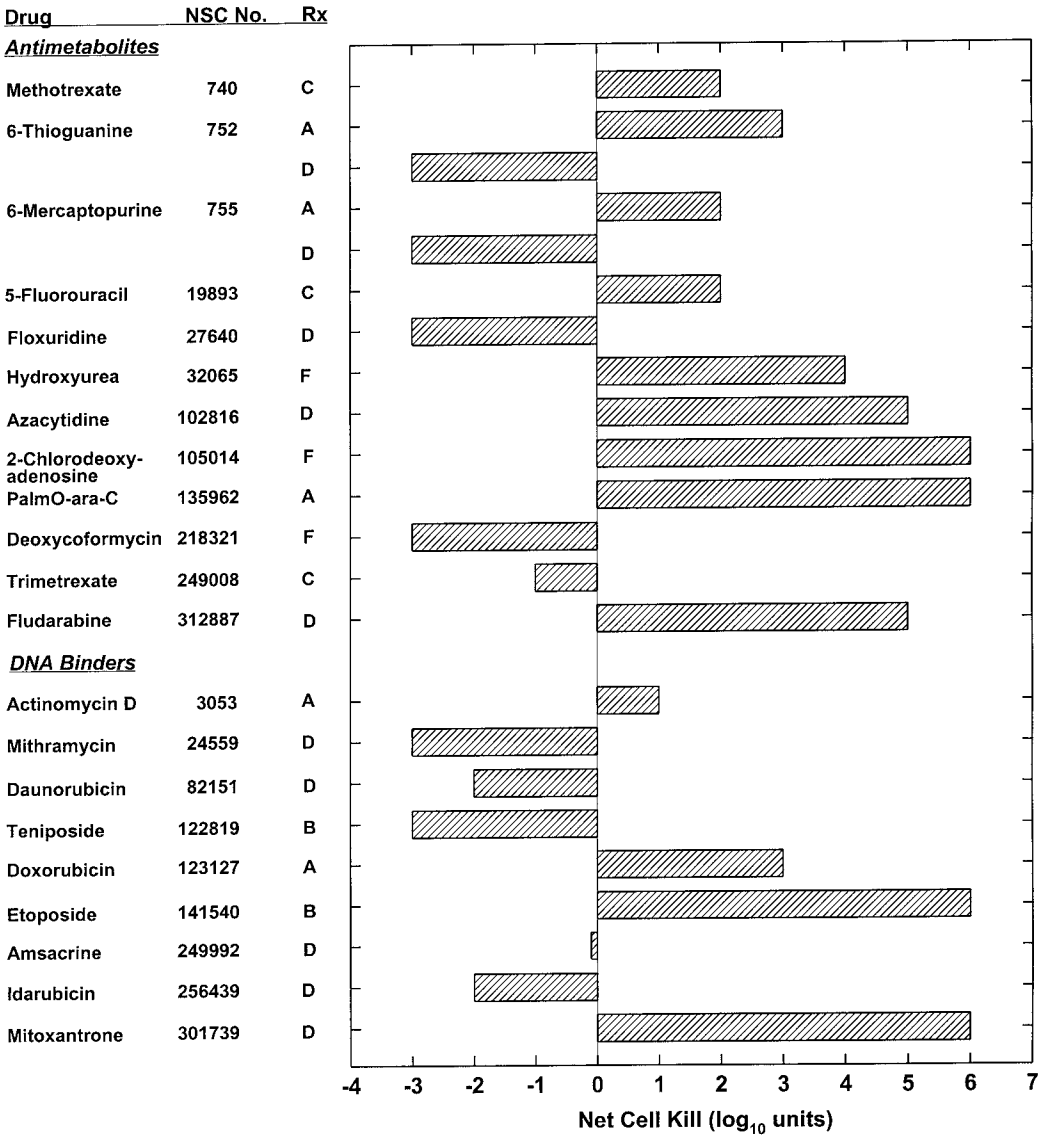

Fig. 2. Sensitivity of ip-implanted L1210 leukemia to clinically useful antimetabolites and DNA binders. L1210 leukemia (10^5 cells, except for hydroxyurea, which used 10^4 cells and 6-thioguanine (d 1-only treatment) and daunorubicin, which used 10^6 cells) was ip-implanted on d 0. Beginning on d 1 (d 2 for daunorubicin), the agents were ip-administered using the indicated schedules. Treatment schedule (R_x): see legend for Fig. 1.

C/palmO-ara-C, with ip administration, exhibit comparable activity against either ip- or ic-implanted leukemias. Cisplatin, cyclophosphamide, ifosfamide, and 6-mercaptopurine (L1210), in addition to melphalan, have reduced activity with an ic implant site. Several agents become inactive with an ic implant site—methotrexate (P388), 5-fluorouracil (5-Fu), floxuridine, actinomycin D, vincristine, doxorubicin, and etoposide. Comparisons among different treatment schedules can be misleading. Although all values have been expressed as net cell-kill (i.e., corrected for the treatment schedule),

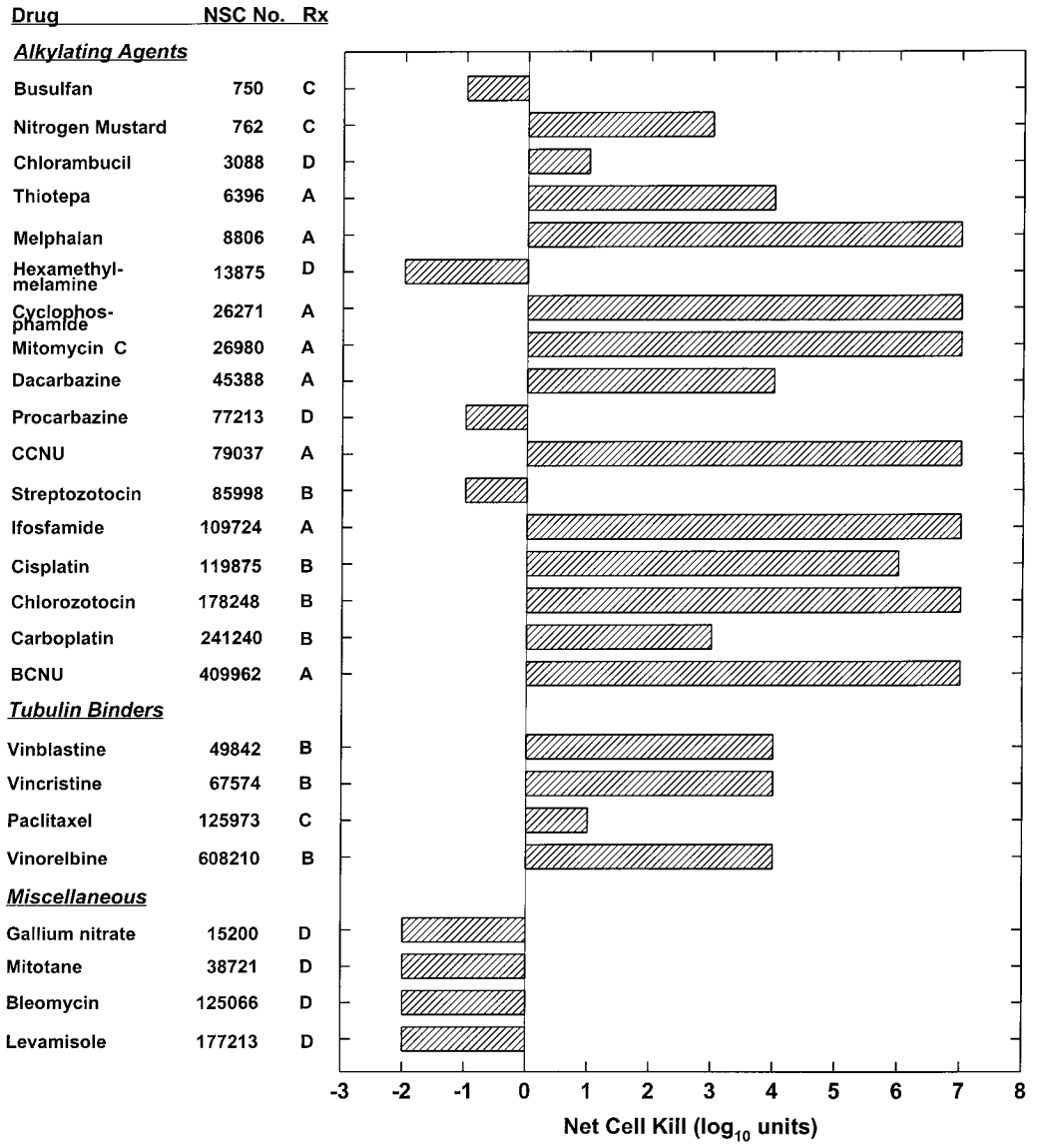

Fig. 3. Sensitivity of ip-implanted P388 leukemia to clinically useful alkylating agents, tubulin binders, and other miscellaneous agents. P388 leukemia (10^6 cells except for CCNU, which used 10^7 cells) was ip-implanted on d 0. Beginning on d 1 (d 2 for CCNU, streptozotocin, and chlorozotocin), the agents were ip-administered using the indicated schedules. Treatment schedule (R_x): see legend for Fig. 1.

one schedule can be optimal, whereas another schedule is suboptimal. For nitrogen mustard, no conclusion can be drawn from the data about its ability to cross the blood-brain barrier. The agent is active against the ip-implanted leukemia using a single ip injection (optimal), and is inactive against the ic-implanted leukemia using 15 daily ip injections (suboptimal). This is further illustrated by chlorambucil, which is active against ic-implanted L1210 (using a single ip injection), and inactive against ip-implanted L1210 (using nine daily ip injections).

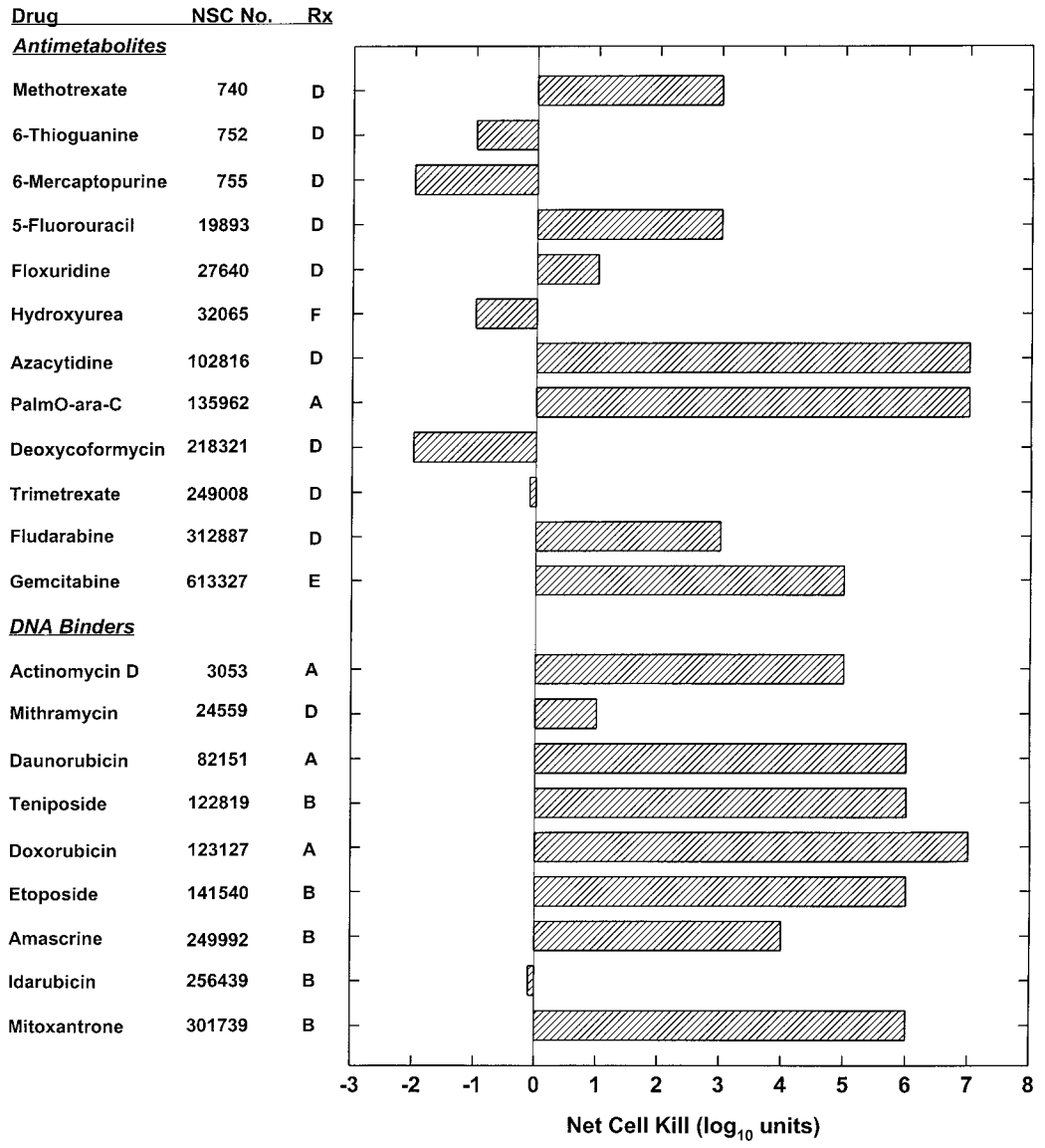

Fig. 4. Sensitivity of ip-implanted P388 leukemia to clinically useful antimetabolites and DNA binders. P388 leukemia (10^6 cells) was ip-implanted on d 0. Beginning on d 1, the agents were ip-administered using the indicated schedules. Treatment schedule (R_x): see legend for Fig. 1.

Studies with these screening models revealed that drug sensitivity was, in some cases, heavily dependent on drug concentration and exposure time, which in turn was impacted by the in vivo treatment schedule. As an example, studies conducted with 1-β-D-arabinofuranosylcytosine (ara-C) pointed out the need for concentration and time of exposure studies. Using L1210 leukemia in mice, it was shown that the optimal dosage and schedule for ara-C was 15–20 mg/kg/dose, given every 3 h for eight doses, then repeated three times at 4-d intervals *(13)*. This regimen was "curative." The single-dose LD_{10} for mice was between 2500 and 3000 mg/kg, and using a single dose within

Table 1
Activity of Melphalan Administered as a Single IP Injection Against L1210 and P388 Leukemias Implanted ip, iv, and ic

		Net cell-kill (log_{10} units)	
Site	*Inoculum size*	*L1210*	*P388*
IP	10^6	4.7	>6.5
IV	10^6	2.0	2.9
IC	10^4	1.2	2.4

that range would effect a 3-log_{10}-unit reduction in L1210 cells but was not "curative." Although these in vivo results might give the appearance of a concentration-dependent effect, in vitro studies have clearly shown that cell-kill of L1210 in culture was time-dependent at the higher concentration levels employed. The apparent concentration dependence observed in vivo over a range of single doses resulted from the extended time of exposure of those extremely high dosage levels.

5. PREDICTIVE VALUE

Many investigators have questioned the use of experimental leukemias as primary screening models over the years. Some have argued that since L1210 or P388 leukemia was used for many years as the initial screening model, continued evaluation of compounds emerging from this screening configuration—even using solid-tumor models for secondary evaluation—would only produce antileukemic drugs *(14)*. If compounds active against solid tumors were being missed by the primary screen composed of leukemias, it would appear reasonable that in order to obtain agents that are active against specific tumor types or solid tumors in general, then the primary screen should consist of specific tumor types or solid tumors. Although this would appear to be a reasonable approach, it will depend on whether or not there are existing agents or whether agents can be developed that will selectively kill specific cancer histotypes.

The correlation between drugs active against L1210 or P388 leukemia and solid experimental tumor models has not been good. For example, only 1.7% of 1493 agents that were active against P388 leukemia were also active against murine LLC. Further, only 2% of 1507 agents active against P388 leukemia were also active against murine colon 38 adenocarcinoma. Finally, only 2% of 1133 agents that were active against P388 leukemia were also active against human CX-1 (HT29) colon tumor. However, when comparing leukemias, a correlation less than expected was obtained—only 15% of 1564 active agents against P388 leukemia were also active against L1210 leukemia *(15)*.

One common observation is that some drugs that are active against experimental solid tumors are inactive against P388 leukemia. For example, 15% of 84 agents that were inactive against P388 leukemia were active against at least one of eight solid tumors tested *(15)*. Flavone acetic acid has been cited as an example *(14)*. This compound was inactive in the initial P388 screen, although it was later shown to exhibit

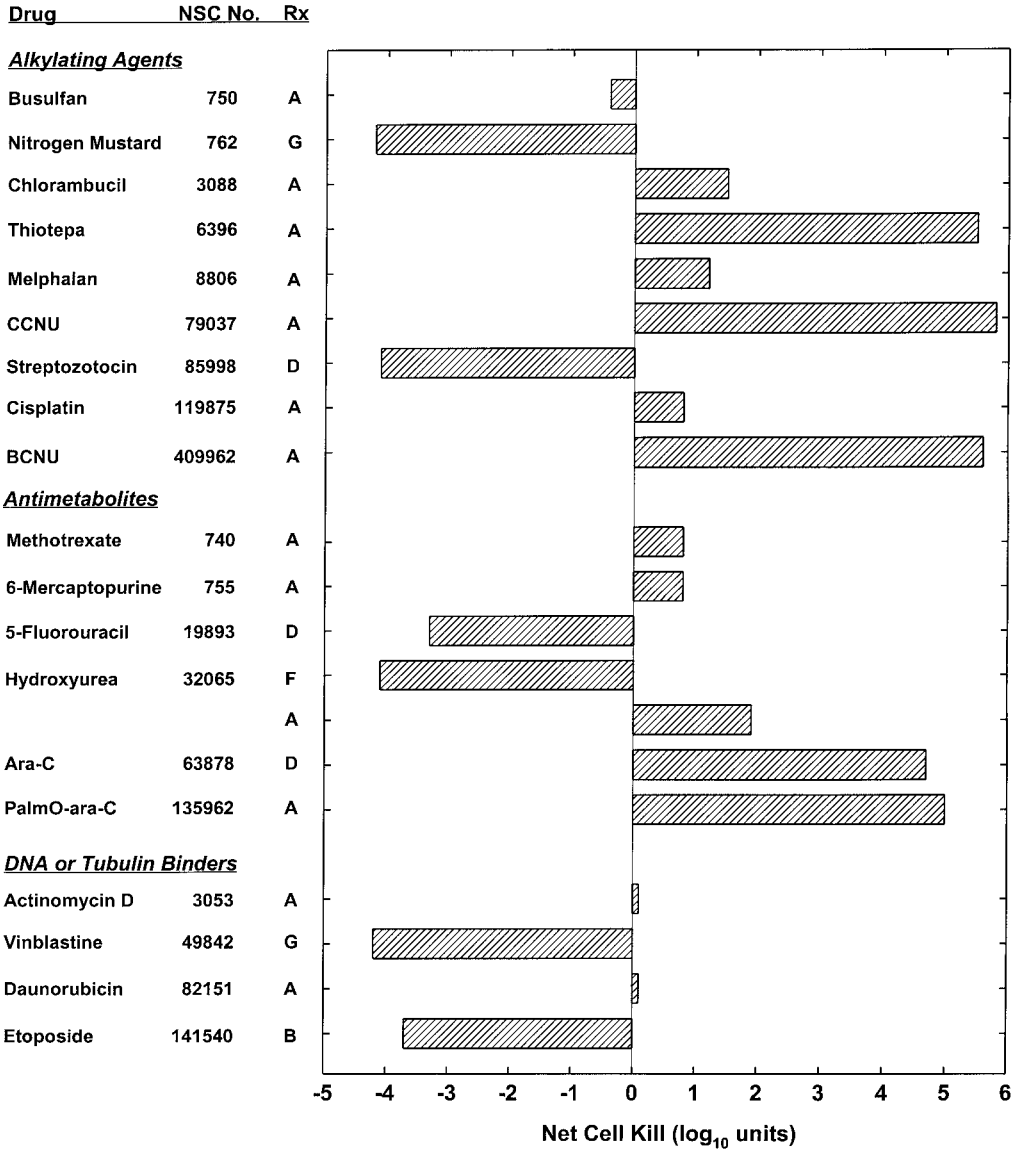

Fig. 5. Sensitivity of ic-implanted L1210 leukemia to clinically useful agents. L1210 leukemia (10^4 cells except for CCNU, which used 10^5 cells) was ic-implanted on d 0. Beginning on d 1 (d 2 for busulfan, chlorambucil, thiotepa, melphalan, hydroxyurea (single injection), cisplatin, BCNU, and daunorubicin), the agents were ip-administered using the indicated schedules. Treatment schedule (R_x): see legend for Fig. 1.

activity against the leukemia when the appropriate treatment schedule was used *(16)*. This example reveals a problem with large-scale screening programs—it is not logistically feasible to conduct preliminary schedule-dependency trials.

Another observation is that there are experimental solid tumors (e.g., murine pancreatic 02 ductal adenocarcinoma) that are not responsive in vivo to any clinically used agents, including many P388-active agents *(14)*. It may be noted, however, that this

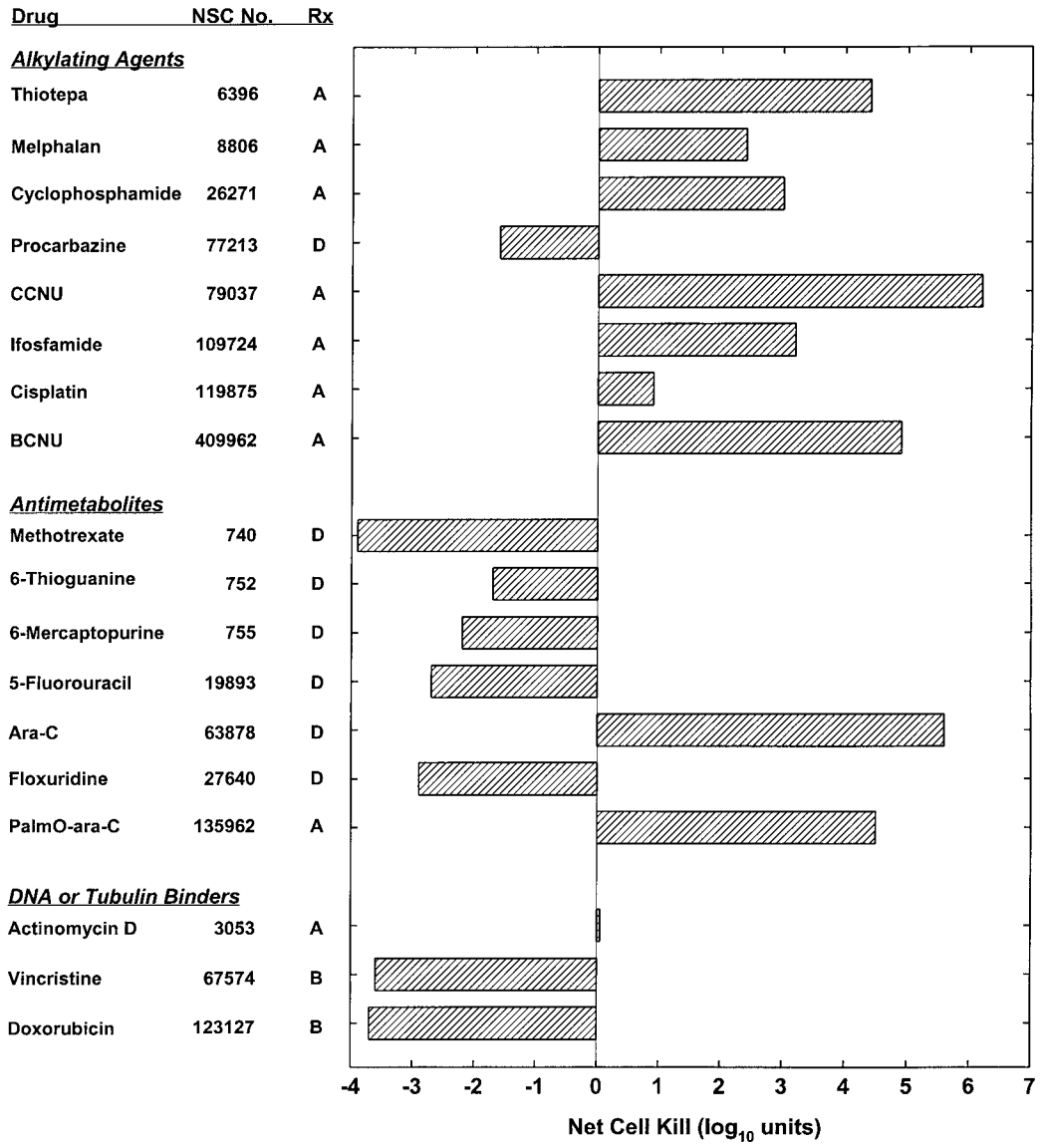

Fig. 6. Sensitivity of ic-implanted P388 leukemia to clinically useful agents. P388 leukemia (10^4 cells except for ifosfamide, methotrexate, 6-thioguanine, 6-mercaptopurine, 5-FU, and floxuridine, which used 10^3 cells and CCNU and ara-C, which used 10^5 cells) was ic-implanted on d 0. Beginning on d 1 (d 2 for ifosfamide), the agents were ip-administered using the indicated schedules. Treatment schedule (R_x): see legend for Fig. 1.

tumor is sensitive to numerous clinical agents in vitro after a 24-h exposure *(17)*, suggesting that the in vivo insensitivity of this tumor may not be caused by cellular characteristics, but may be a result of physiological or architectural constraints of the animal.

Southern Research has evaluated a spectrum of compounds in the ip-implanted P388 model in order to evaluate this model as a predictor for the response of human tumor xenografts to new candidate antitumor agents (unpublished data). The P388 data col-

lected were compared to the data for various sc-implanted human tumor xenografts, which were selected on the basis of the results of the NCI in vitro screen. In general, compounds that were active against P388 leukemia were active to a lesser degree in one or more of the xenografts in the in vivo tumor panel. However, there were isolated examples of a P388-active agent being inactive in the human tumor xenograft models tested, and vice versa. There was no indication that the P388 model could predict compound efficacy for specific tumor xenografts.

Whether or not the murine leukemias are poor predictors of activity in solid tumors is still somewhat questionable, and will only be determined with the availability of drugs without antileukemic model activity but with proven value in the treatment of human solid tumors.

6. DRUG-RESISTANT LEUKEMIAS

Panels of in vivo drug-resistant murine L1210 and P388 leukemia models have been developed at Southern Research for use in the evaluation of crossresistance and collateral sensitivity. These models have been used for the evaluation of new drugs of potential clinical interest. An extensive summary of in vivo drug resistance and crossresistance data has been published by Schabel and colleagues *(18)*. Their initial manuscript included results of in vivo crossresistance studies on 79 antitumor drugs in seven drug-resistant L1210 leukemias and 74 antitumor drugs in 12 drug-resistant P388 leukemias. Previously, we expanded this crossresistance data base for the drug-resistant P388 leukemias to include two new drug-resistant lines and more clinically useful drugs. Also, we updated the database to include new candidate antitumor agents entering clinical trials *(19)*. Recently, another drug-resistant P388 leukemia (P388/VP-16) was added to this database *(20)*. This section examines the crossresistance database for 16 drug-resistant P388 leukemias and many of the clinically useful agents.

6.1. Resistance to Alkylating Agents

The crossresistance profile of cyclophosphamide-resistant P388 leukemia (P388/CPA) to 14 different clinical agents is shown in Table 2. The P388/CPA line was crossresistant[1] to one of the five alkylating agents, no antimetabolites, no DNA-binding agents, and no tubulin-binding agents. Crossresistance of P388/CPA has also been observed for two other alkylating agents (chlorambucil and ifosfamide) *(20)*. Interestingly, there are differences among these three agents. Chlorambucil and ifosfamide, like cyclophosphamide, each have two chloroethylating moieties, whereas mitomycin C is from a different chemical class. Whereas ifosfamide, cyclophosphamide, and mitomycin C require metabolic activation, chlorambucil does not. Although P388/CPA is crossresistant to two chloroethylating agents, the line is not crossresistant to other chloroethylating agents (melphalan and BCNU). Therefore, P388/CPA appears to be crossresistant only to a select group of alkylating agents with differing characteristics. P388/CPA appeared to be collaterally sensitive to fludarabine.

[1] Crossresistance is defined as decreased sensitivity (by >2-$\log_{10}$ units of cell-kill) of a drug-resistant P388 leukemia to a drug compared to that observed concurrently in P388/0 leukemia. Similarly, marginal crossresistance is defined as a decrease in sensitivity of approx 2-$\log_{10}$ units. Collateral sensitivity is defined as increased sensitivity (by >2-$\log_{10}$ units of cell kill) of a drug-resistant P388 leukemia to a drug over that observed concurrently in P388/0 leukemia.

Table 2
Crossresistance of P388 Sublines Resistant to Various Alkylating Agents and Antimetabolites to Clinically Useful Agents

Drug	*NSC No.*	*R_x*[a]	*CPA*	*L-PAM*	*DPt*	*BCNU*	*MMC*[b]	*MTX*	*5-FU*	*ARA-C*
Alkylating Agents										
Melphalan	8806	A	–	+	–	–				±
Cyclophosphamide	26271	A	+	–	–	–	–			+
Mitomycin C	26980	A	±	+	–	–	+	–		+
Procarbazine	77213	D	–							
Cisplatin	119875	B	–	+	+	–	±[c]	–		+
BCNU	409962	A	–	–	–	+	–			–
Antimetabolites										
Methotrexate	740	D			–		–[d]	+	+	+
6-Thioguanine	752	A					–[d]	–		
6-Mercaptopurine	755	D						–		
5-Fluorouracil	19893	D	–		–		–[d]		+	=
PalmO-ara-C	135962	A	–	–	–		–[d]	–	–	+
Trimetrexate	249008	D		±	–			–		–
Fludarabine	312887	D	=	=	=			–	=	+
Gemcitabine	613327	E	–	–	–			–		+
DNA Binders										
Actinomycin D	3053	A	–	±	–		±			
Doxorubicin	123127	A	–	–	–		+	–		–
Etoposide	141540	B	–	–	–		+[c]	–		–
Amsacrine	249992	B	–	+	=		–[c]	–		–
Mitoxantrone	301739	B		+	=			–		–
Tubulin Binders										
Vinblastine	49842	A					+			
Vincristine	67574	B	–	+	–		+[c]	–		+
Paclitaxel	125973	C		–	–			–		–

$CD2F_1$ mice were ip-implanted with 10^6 P388/0 or drug-resistant P388 cells on d 0. Data presented are for ip drug treatment at an optimal ($\leq LD_{10}$) dosage. Symbols: resistance/crossresistance, +; marginal crossresistance, ±; no crossresistance, –; and collateral sensitivity, =.

[a] Treatment schedule (R_x): A = d 1; B = d 1, 5, 9; C = d 1–5; D = d 1–9; E = d 1, 4, 7, 10.

[b] Data from *In Vivo* 1987; 1:47–52.

[c] Treatment schedule was d 1.

[d] Treatment schedule was d 1 and 5.

The effect of 15 different clinical agents on melphalan-resistant P388 leukemia (P388/L-PAM) is shown in Table 2. The P388/L-PAM line was crossresistant to approximately one-half of the agents—2 of 4 alkylating agents, 1 of 4 antimetabolites, 3 of 5 DNA-binding agents, and 1 of 2 tubulin-binding agents. The alkylating agents involved in crossresistance represent different chemical classes. Similarly, the DNA-interacting agents involved in crossresistance include agents with different mechanisms of action—inhibitors of DNA topoisomerase II (amsacrine and mitoxantrone) and a DNA-binding agent (actinomycin D). However, the melphalan-resistant line did not exhibit crossresistance to other inhibitors of DNA topoisomerase II (e.g., doxorubicin and etoposide) or another DNA-binding agent (e.g., doxorubicin).

The sensitivity of cisplatin-resistant P388 leukemia (P388/DDPt) to 17 different clinical agents is shown in Table 2. The P388/DDPt line was not crossresistant to any of these agents. Interestingly, the cisplatin-resistant line was collaterally sensitive to three agents (fludarabine, amsacrine, and mitoxantrone). Of these three agents, the latter two have been reported to interact with DNA topoisomerase II *(21,22)*.

The crossresistance data for N,N′-*bis*(2-chloroethy1)-N-nitrosourea-resistant P388 leukemia (P388/BCNU) have been limited to the evaluation of alkylating agents. The crossresistance profile of P388/BCNU to four different clinical agents is shown in Table 2. The BCNU-resistant line was not crossresistant to melphalan, cyclophosphamide, mitomycin C, or cisplatin.

The crossresistance profile of mitomycin C-resistant P388 leukemia (P388/MMC) to 13 different clinical agents is shown in Table 2 *(23)*. The P388/MMC line was crossresistant to approximately one-half of the agents—1 of 3 alkylating agents, 0 of 4 antimetabolites, 3 of 4 DNA-binding agents, and two of two tubulin-binding agents. The pattern was similar to that observed for P388/L-PAM.

6.2. Resistance to Antimetabolites

The effect of 14 different clinical agents on methotrexate-resistant P388 leukemia (P388/MTX) is shown in Table 2. The P388/MTX line was not crossresistant to any of these agents.

The crossresistance data for 5-fluorouracil-resistant P388 leukemia (P388/5-FU) have been limited to antimetabolites. The sensitivity of the P388/5-FU to three different agents is shown in Table 2. The P388/5-FU line was not crossresistant to palmO-ara-C (a slow-releasing form of ara-C) or fludarabine (possible collateral sensitivity). Crossresistance was observed for methotrexate.

The crossresistance profile of 1-β-D-arabinofuranosylcytosine-resistant P388 leukemia (P388/ARA-C) to 16 different clinical agents is shown in Table 2. The P388/ARA-C line was crossresistant to members of several functionally different classes of antitumor agents—four of five alkylating agents, three of five antimetabolites, none of four DNA-binding agents, and one of two tubulin-binding agents. Interestingly, the line was collaterally sensitive to 5-FU.

6.3. Resistance to DNA- and Tubulin-Binding Agents

The effect of 17 different clinical agents on actinomycin D-resistant P388 leukemia (P388/ACT-D) is shown in Table 3. P388/ACT-D was not crossresistant to any alkylating agents or antimetabolites. However, it was crossresistant to all of the drugs tested that are involved in multidrug resistance, except for amsacrine.

Table 3
Crossresistance of P388 Sublines Resistant to Various DNA and Tubulin Binders to Clinically Useful Agents

Drug	*NSC No.*	R_x[a]	*ACT-D*	*ADR*	*AMSA*	*DIOHA*	*VP-16*	*CPT*[b]	*VCR*	*PTX*
Alkylating Agents										
Melphalan	8806	A	–	–	–		–		–	
Cyclophosphamide	26271	A	–	–	–	–	–		–	
Mitomycin C	26980	A		±	–			–[e]	+	
Procarbazine	77213	D	–	–					–	
Cisplatin	119875	C	–	–	–[c]		–[c]	–[e]	±	
BCNU	409962	A	–	–			–		–	
Antimetabolites										
Methotrexate	740	D	–	–	±		–		–	
6-Thioguanine	752	D	–	–					–	
6-Mercaptopurine	755	D	–	–					–	
5-Fluorouracil	19893	D	–	–[d]	–[d]		–		–	
PalmO-ara-C	135962	A	–	–	–				–	
Trimetrexate	249008	D		–					–	
Fludarabine	312887	D		=					–	
Gemcitabine	613327	E		–			–		–	
DNA Binders										
Actinomycin D	3053	A	+	+	+	–	+	–[e]	–	
Doxorubicin	123127	A	±	+	+	–	+	–[e]	–	+
Etoposide	141540	B	+	+	+	–	+		–	+
Amsacrine	249992	B	–	+	+	+	+	–[e]	–	
Mitoxantrone	301739	B	+	+	+	+	+	–[e]	–	
Tubulin Binders										
Vinblastine	49842	B	+	+	+		+		+	
Vincristine	67574	B	+	+	+	+	+		+	+
Paclitaxel	125973	C	±	±	+	–		–[e]	–	+

$CD2F_1$ mice were ip-implanted with 10^6 P388/0 or drug-resistant P388 cells on d 0. Data presented are for ip drug treatment at an optimal ($\leq LD_{10}$) dosage. Symbols: resistance/crossresistance, +; marginal crossresistance, ±; no crossresistance, –; and collateral sensitivity, =.

[a] Treatment schedule (R_x): A = d 1; B = d 1, 5, 9; C = d 1–5; D = d 1–9; E = d 1, 4, 7, 10.

[b] Data from *Mol Pharmacol* 1990; 38:471–480.

[c] Treatment schedule was d 1, 5, 9.

[d] Treatment schedule was d 1–5.

[e] Treatment schedule was d 1 and 5.

The crossresistance profile of doxorubicin-resistant P388 leukemia (P388/ADR) to 21 different clinical agents is shown in Table 3. The P388/ADR line was not crossresistant to any of the antimetabolites, and was marginally crossresistant to only one alkylating agent (mitomycin C). Resistance was observed for all the drugs tested that are reported to be involved in multidrug resistance (actinomycin D, doxorubicin, etoposide, amsacrine, mitoxantrone, vinblastine, vincristine, and paclitaxel). P388/ADR was collaterally sensitive to fludarabine.

The sensitivity of amsacrine-resistant P388 leukemia (P388/AMSA) to 14 different clinical agents is shown in Table 3. P388/AMSA was not crossresistant to any of the alkylating agents, and was marginally crossresistant to only one antimetabolite. Crossresistance was observed for all the drugs tested that are involved in multidrug resistance.

The crossresistance data for mitoxantrone-resistant P388 leukemia (P388/DIOHA) have been limited mainly to agents involved in multidrug resistance. The sensitivity of P388/DIOHA to seven different clinical agents is shown in Table 3. The P388/DIOHA line exhibited mixed multidrug resistance—crossresistance to amsacrine and vincristine, but no crossresistance to actinomycin D, doxorubicin, etoposide, or paclitaxel.

The crossresistance profile of etoposide-resistant P388 leukemia (P388/VP-16) to 13 different clinical agents is shown in Table 3. The P388/VP-16 line was not crossresistant to any of the alkylating agents or antimetabolites. However, it was crossresistant to all of the drugs tested that are reported to be involved in multidrug resistance.

The sensitivity of camptothecin-resistant P388 leukemia (P388/CPT) to seven different clinical agents is shown in Table 3 *(24)*. P388/CPT was not crossresistant to any of these agents.

The effect of 21 different clinical agents on vincristine-resistant P388 leukemia (P388/VCR) is shown in Table 3. The P388/VCR line was crossresistant to three of the agents—mitomycin C, cisplatin (marginal), and vinblastine. Unexpectedly, P388/VCR was not crossresistant to many of the drugs tested that are involved in multidrug resistance (e.g., actinomycin D, doxorubicin, etoposide, amsacrine, mitoxantrone, and paclitaxel).

The crossresistance data for paclitaxel-resistant P388 leukemia (P388/PTX) have been limited to agents involved in multidrug resistance. The sensitivity of P388/PTX to three different clinical agents is shown in Table 3. The P388/PTX line was crossresistant to drugs that are involved in multidrug resistance (doxorubicin, etoposide, and vincristine).

CONCLUSION

Currently, biotechnology appears to be advancing in an almost exponential fashion. Today, advanced techniques and tools allow us to conduct research that could not even be imagined 40 yr ago, when the L1210 and P388 leukemia models were first used extensively (e.g., sequencing the human genome). Utilizing molecular biology techniques, the emphasis is now on the development of compounds designed for a specific target. Current NCI strategy suggests that models for evaluating these compounds contain the specific target, either naturally or by gene transfection. Successful treatment of such models will theoretically provide the necessary proof-of-concept required for continued development. This is a radical departure from the empirical approach to mass screening of compounds against murine leukemias.

The L1210 and P388 leukemia models do have some advantages—they are rapid, reproducible, and relatively inexpensive (in comparison to human tumor xenograft models). However, as with any experimental animal tumor model, there are limitations. Neither leukemia is a satisfactory drug discovery model for either human cancer in general or human leukemia in particular. Of course, this could be said of any animal tumor model. Of the two leukemias, P388 is the more sensitive, but overpredicts drug activity for both preclinical human tumor xenograft models and the clinic. However, the question of whether P388 leukemia (or L1210) is a poor predictor for solid tumor-active drugs has not yet been sufficiently answered.

Although the murine leukemia models have serious limitations, these models have been very useful in anticancer drug development, in the development of a number of therapeutic principles, and in understanding the biologic behavior of tumor and host. These models are still useful today in conducting detailed evaluations of new candidate anticancer drugs (e.g., schedule dependency, route-of-administration dependency, formulation comparison, analog comparison, and combination chemotherapy).

Perhaps the greatest utility of the murine leukemias today is derived from the evaluations of the drug-resistant sublines for crossresistance and collateral sensitivity. Analysis of the crossresistance data generated at Southern Research for clinical agents has revealed possible noncrossresistant drug combinations. The P388 leukemia lines selected for resistance to alkylating agents (e.g., P388/CPA, P388/L-PAM, P388/DDPt, P388/BCNU, and P388/MMC) differed in crossresistance profiles, both with respect to alkylating agents and other functional classes. Similarly, P388 leukemia lines selected for resistance to antimetabolites (e.g., P388/MTX, P388/5-FU, and P388/ARA-C) differed in crossresistance profiles, both with respect to antimetabolites and other functional classes. Clearly, the spectrum of crossresistance of an alkylating agent or an antimetabolite will depend on the individual agent. P388 leukemia lines selected for resistance to large polycyclic anticancer drugs (e.g., P388/ACT-D, P388/ADR, P388/AMSA, P388/DIOHA, P388/VP-16, P388/CPT, P388/VCR, and P388/PTX) were not generally crossresistant to alkylating agents or antimetabolites. However, the crossresistance profiles to DNA- and tubulin-binding agents were variable.

Five of the 16 drug-resistant leukemias exhibited collateral sensitivity to one or more drugs. These observations of collateral sensitivity suggest that a combination of one of the five drugs plus one of the corresponding agents for which collateral sensitivity was observed may exhibit therapeutic synergism.

Crossresistance data, coupled with knowledge of the mechanisms of resistance operative in the drug-resistant leukemias, may yield insights into the mechanisms of action of the agents being tested. Similarly, crossresistance data, coupled with the mechanisms of action of various agents, may yield insights into the mechanisms of resistance operative in the drug-resistant leukemias *(19)*. Furthermore, crossresistance data may identify potentially useful guides for patient selection for clinical trials of new antitumor drugs *(19)*.

In conclusion, the role of L1210 and P388 leukemias in the evaluation of anticancer agents has diminished considerably. Nevertheless, the majority of clinical agents now in use was first detected by the murine leukemias. These models are clearly still appropriate for answering certain questions, and the drug-resistant sublines can provide valuable information concerning crossresistance and collateral sensitivity.

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