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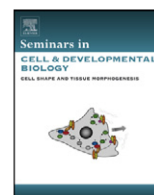

Review

# Lead genetic studies in *Dictyostelium discoideum* and translational studies in human cells demonstrate that sphingolipids are key regulators of sensitivity to cisplatin and other anticancer drugs

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ABSTRACT

A *Dictyostelium discoideum* mutant with a disruption in the sphingosine-1-phosphate (S-1-P) lyase gene was obtained in an unbiased genetic analysis, using random insertional mutagenesis, for mutants with increased resistance to the widely used cancer chemotherapeutic drug cisplatin. This finding opened the way to extensive studies in both *D. discoideum* and human cells on the role and mechanism of action of the bioactive sphingolipids S-1-P and ceramide in regulating the response to chemotherapeutic drugs. These studies showed that the levels of activities of the sphingolipid metabolizing enzymes S-1-P lyase, sphingosine kinase and ceramide synthase, affect whether a cell dies or lives in the presence of specific drugs. The demonstration that multiple enzymes of this biochemical pathway were involved in regulating drug sensitivity provided new opportunities to test whether pharmacological intervention might increase sensitivity. Thus it is of considerable clinical significance that pharmacological inhibition of sphingosine kinase synergistically sensitizes cells to cisplatin, both in *D. discoideum* and human cells. Linkage to the p38 MAP kinase and protein kinase C (PKC) signaling pathways has been demonstrated. This work demonstrates the utility of *D. discoideum* as a lead genetic system to interrogate molecular mechanisms controlling the sensitivity of tumor cells to chemotherapeutic agents and for determining novel ways of increasing efficacy. The *D. discoideum* system could be easily adapted to a high throughput screen for novel chemotherapeutic agents.

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## 1. Introduction: chemotherapy and cancer

Of the roughly 1.5 million people diagnosed with cancer in the United States this year, many will receive chemotherapy at some time during the course of their treatment [1]. Conventional chemotherapy generally consists of the systemic administration of one or more cytotoxic drugs that are known to kill the rapidly dividing cancer cells. Despite its widespread use, conventional chemotherapy suffers from significant problems including lack of specificity towards the tumors (compared to normal cells) and a significant frequency of tumors that are, or become, resistant to the chemotherapeutic drugs. Major efforts are being made to rationally design drugs that will specifically stop tumor growth by inhibiting tumor specific targets such as oncoproteins, or activating quiescent cell death pathways in tumor cells [2–4]. Although there have been some elegant successes, they are limited to a small subset of cancers, and major advances in drug design that will impact the entire spectrum of tumor types are still well in the future. Thus, there is a

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continuing need to better understand the mechanisms underlying resistance to conventional chemotherapeutic drugs, and thereby devise ways to make these drugs more effective.

In this regard, there has been a great deal of research in the area of anti-cancer drug resistance and this effort continues today. Primarily, this work has been done on cultured mammalian (human and mouse) cells and often on cell lines derived from tumors. The work has often been focused on “candidate targets” which are generally proteins believed *a priori* to be involved in affecting the sensitivity to drugs – for example proteins involved in the processes of drug influx or efflux, drug detoxification, DNA damage repair, and regulating cell proliferation or cell death. An examination of the thousands of published papers in this area shows that there is considerable confusion regarding the underlying mechanisms that determine sensitivity and resistance. These investigations are hampered by a number of issues including: (1) different cell lines which can have dramatically different origins and properties; (2) genetic abnormalities in cultured cell lines, including vast differences in ploidy; (3) efforts to select for drug resistant cell lines by incrementally increasing drug concentration, but where it is ultimately impossible to make single gene mutations, to clonally isolate the mutant lines, or to identify the cognate mutated genes; and (4) the lack of defined protocols for testing drug sensitivity. Overall, the problem of resistance to chemotherapeutic drugs can benefit from analysis in a genetic system in which single isogenic mutations can be easily isolated and analyzed.

## 2. *Dictyostelium discoideum*: its biochemistry, cell biology and genetics make it a good target for drugs and understanding drug resistance

The simple eukaryote *D. discoideum* has been studied extensively since its discovery in 1935 [5] as a model for multicellular morphogenesis and cytodifferentiation using a combination of genetic, biochemical and molecular methods. It has an interesting life cycle, in which cells divide mitotically and remain single until they deplete their nutrient supply, at which time they aggregate using chemotaxis into multicellular assemblies (tissues) each consisting of $10^5$ cells. The multicellular assemblies then proceed synchronously through the program of development, whereby they each produce a single fruiting body consisting of a mass of 80,000 spores resting atop a multicellular stalk of 20,000 cells [6]. Thus, thousands of identically staged “embryos” can be obtained in a single Petri dish for biochemical and molecular analyses. When presented with nutrients, each spore can germinate into a single cell, which again begins to divide mitotically and ultimately can aggregate with other cells and undergo development. These mitotically dividing cells behave, and can be handled, like any other cultured animal cell. *D. discoideum* cells are eukaryotic with a simple plasma membrane (no cell wall) and an intracellular architecture typical of mammalian cells. They grow rapidly in inexpensive medium and clonal cell lines derived from single cells can easily be isolated [7].

The genome of *D. discoideum* has been completely sequenced, gene expression studies have been performed, and annotation continues as new data are published ([8,9]; and www.dictybase.org). There are approximately 12,000 coding sequences, relatively little gene redundancy, and numerous genes are homologous to those known to be involved in human disease [8,10]. For example, there are homologs of the human nucleotide excision repair (xeroderma pigmentosum) genes [11–14], the base excision repair genes [15,16], and non-homologous end-joining genes [17,18] that play a prominent role in preserving genome integrity, and when mutated contribute to cancer.

Well-established methods are available for the knock-in and knock-out of genes by direct homologous recombination [13,19], and random insertional mutagenesis (called REMI for *restriction enzyme mediated integration*) [20]. The availability of the entire genome sequence and the development of these molecular methods had led to a surge of detailed analyses of the biology of this organism in the past decade.

There has been a long history of genetic analysis of *D. discoideum* and it is tightly interwoven with the analysis of resistance to drugs and other toxic chemicals. The organism is haploid which allows the facile isolation of mutants, and there is a parasexual genetic system for mapping mutations to chromosomes, and for the determination of complementation and dominance [21]. Mutations for resistance to methanol, acraflavin, cycloheximide, ethidium bromide, cobalt chloride and some microtubule inhibitors were isolated using chemical mutagens and were then used as chromosome markers in genetic analyses [22]. With the advent of insertional mutagenesis, some of these mutants have been re-examined, and the molecular basis for the phenotypes was elucidated. Such was the case for methanol and acraflavin resistance where the resistant phenotypes were shown to be due to specific loss-of-function mutations in the catalase A gene [23]. These studies established *D. discoideum* as an appropriate model for a blind unbiased genetic analysis of the molecular basis of resistance to chemotherapeutic drugs [24,25].

## 3. Selecting drug resistant mutants

The drug cisplatin (*cis*-diamminedichloroplatinum (II)) is widely used to treat non-Hodgkin's lymphoma, small cell and non-small cell lung, testicular, ovarian, head and neck, esophageal, and bladder cancer [26]. It is a small molecular weight molecule that primarily forms covalent adducts with adjacent purines on DNA [27]. Its anticancer effect is widely believed to be due to this chemistry, although there is a generally poor understanding of the subsequent signaling pathways that regulate its action. Its therapeutic efficacy is often limited by its general cytotoxicity as well as by the fact that tumors become resistant to the drug [28]. Cisplatin is used at its maximally allowable dose so increasing the concentration to overcome resistance is not an option, and efforts to synthesize more potent cisplatin analogs have not been greatly successful [29]. The literature on resistance to cisplatin is vast (over 5400 references) and no consensus has emerged regarding the underlying mechanism(s), which are clearly complex and probably multifactorial. Many of these studies were performed on drug resistant cell lines, which contain multiple mutations and aberrant chromosome numbers and structures. Moreover, in most cases a protein suspected of causing resistance was the subject of the study.

Motivated by these issues that make genetic analysis in mammalian cells difficult, a blind genetic selection for cisplatin resistant mutants was performed using *D. discoideum*. The goal was to obtain single gene mutants, in possibly unsuspected pathways, that could be further analyzed using genetics and biochemistry. The random insertional mutagenesis and selection resulted in 7 clonal cisplatin resistant lines, each with a single gene disruption [30]. The disrupted genes were cloned and sequenced. Homologous recombination was used to regenerate targeted null mutations in these genes, and these mutants had the same cisplatin resistant phenotype as the original insertional mutants. None of the identified genes had ever been shown previously to have a role in regulating cisplatin sensitivity and thus represented novel targets that might be ultimately used to increase therapeutic efficacy. None of the novel gene products functioned in drug uptake or efflux, detoxification, or DNA repair – the candidate processes that had often been the focus of studies due to their perceived role in regulating cisplatin sensitivity. The random mutagenesis was estimated

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