Macrophage Cytological Profiling and Anti-Inflammatory Drug Discovery

Tannia A. Lau, Walter M. Bray, and R. Scott Lokey

Department of Chemistry and Biochemistry, University of California Santa Cruz, Santa Cruz, California.

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ABSTRACT

Millions of people are affected by diseases and conditions related to the immune system. Unfortunately, our current supply of approved anti-inflammatory medicine is very limited and only treats a small fraction of inflammatory diseases. Nearly half of the drugs on the market today are natural products and natural product derivatives. The long-term objective of my research is to continue efforts toward the discovery of diverse chemical compounds and their mechanism of action (MOA) to inspire the next generation of novel therapeutics. This project approaches this objective by creating a robust platform for the in-depth phenotypic profiling of complex natural product samples with respect to their effect on pathways related to the innate immune response. This approach has the potential to elucidate the MOAs of novel natural products relevant to inflammation and accelerate the pace of drug discovery in this therapeutic area.

Keywords: imaging, immunology, natural products, activation, assay

INTRODUCTION

Natural products are rich sources for anti-inflammatory agents. There are numerous studies quantifying their effects in cell-based model systems as well as whole organisms.^{1–5} Therefore, understanding anti-inflammatory effects of commonly used natural products in folk medicine is warranted. This research will provide a platform for the chemical characterization and mode of action prediction for bioactive natural products in complex mixtures directly from primary screens. Natural products remain a rich source of structural and chemical diversity that is unsurpassed by any synthetic libraries.⁶ Al-

though, there are ~200,000 compounds in the Dictionary of Natural Products, only a small percentage has received biological evaluation to define their mechanism of action (MOA) against prokaryotic or eukaryotic targets.⁷ This lack of biological information is due to at least in part to the fact that there are no well-established high-throughput screening methods for the direct prediction of compound MOA. To address this gap, we utilize cytological profiling (CP), a technology that broadly characterizes the effects of compounds on cells and has the ability to classify cellular phenotypes into clusters of compounds with distinct MOAs.^{8,9} CP is a high-content image-based screen to quantify the phenotypic effects of compounds on mammalian cells by capturing hundreds of cellular features using automated fluorescence microscopy. Utilizing biologically relevant fluorescent markers and computer-aided image segmentation and feature extraction provides detailed quantitative phenotypic profiles on individual cells. The aggregate of processed features gives rise to a “fingerprint” for each compound followed by hierarchical clustering. It is possible to predict the MOAs of unknown compounds^{10,11} by comparing the generated fingerprint to the fingerprints from a training library of ~3000 compounds with known mechanisms of action. Cell images are substantially richer in data compared with single readout, target-based, or cell reporter approaches.^{12–14} Our rationale is to create a system that inverts the natural product drug discovery process, with compound modes of action being determined at the first stage of project selection, rather than as the final step in the drug discovery process.

THE TECHNICAL CHALLENGE

There are currently few general methods for the broad biological characterization of natural products. Current biological annotation tools include the NCI 60-cell line program (National Institutes of Health), which is typically limited to the evaluation of pure compounds with proven efficacy in *in vitro* models; and the Connectivity Map (Broad Institute), which provides genome-wide transcriptional expression data for the set of ~1300 compounds under a variety of experimental conditions and time points. Although powerful, neither of these tools is appropriate for the large-scale analysis of complex natural product libraries, due to the cost and logistical complexities of acquiring data on large (>10,000) screening libraries. By contrast, CP has the

capacity to evaluate many thousands of samples (including crude extracts, prefractions, high performance liquid chromatography fractions, and pure compounds), and is configured to incorporate new data on a rolling basis, with the resolution and accuracy of MOA predictions improving as the size of the data set increases.

Current CP methods utilize cancer cell lines and cytological stains that recognize cell cycle, organelle, and cytoskeletal features.^{9,15–24} Although powerful to query MOAs, many compounds such as bioactive lipids (known to cause pro- and anti-inflammatory effects) and kinase inhibitors show no phenotype in the HeLa cervical cancer cell line using the current stain set. Our original application of CP in HeLa cells revealed that only ~30% of the reference library showed any discernable phenotypic effect (up to 10 μ M and upon 24-h treatment). Thus, compounds that engage and inhibit specific signaling pathways involved in disease states such as inflammation may not give rise to a cytological profile in a resting cancer cell. To address this issue, we will harness the phenotypic plasticity of macrophages under proinflammatory stimulus to uncover new bioactivities in compounds that showed minimal effect in resting cells. Lipopolysaccharide (LPS) is a general name for a class of molecules with a lipid A core that is found in the outer membrane of gram-negative bacteria. LPS initiates the innate immune response by binding Toll-like receptor 4, activating a cascade of intracellular inflammatory signaling pathways.²⁵ Although this response is advantageous as a protection against bacterial infection, it can lead to an uncontrolled release of cytokines and rapid death by septic shock. We hypothesize that LPS-stimulated macrophages, which show a dramatic visual phenotype compared with unstimulated cells, could provide an opportunity for capturing more diverse phenotypes and will improve the ability to distinguish MOA subcategories in CP. Improving CP in this manner could be an effective approach to diversifying our current selection of anti-inflammatories.

IMMUNO-CP: A METHOD TO SCREEN FOR NOVEL THERAPEUTICS FROM NATURAL PRODUCTS TO IDENTIFY ANTI-INFLAMMATORIES

Our laboratory has shown that CP in HeLa cells is a valuable tool to give insights about the potential MOA of lead compounds at the primary screening stage based on a limited staining set that probes the cell cycle, organelles, and the cytoskeleton. Current CP methods utilize cancer cell lines; however, these cell lines exhibit limited phenotypic/functional characteristics in comparison with activated macrophage cells, which exhibit dramatic changes in their form and physiology under inflammatory conditions. We hypothesize that expand-

ing the cell line from HeLa to macrophages with an addition of proinflammatory stimuli, LPS, will improve the ability to assign biological functions to natural product extracts. We have optimized murine macrophage-like RAW264.7 cell conditions with proinflammatory, LPS, and introduced drugs from our known bioactive compound libraries. We have shown the feasibility to apply RAW264.7 macrophages to the CP platform that exhibit measureable differences in cellular features between +LPS and -LPS macrophages. Preliminary data show significant phenotype changes with proinflammatory responses to LPS. We expect that with the addition of the LPS stimulus, we will refine the ability of CP to cluster compounds that engage and inhibit specific signaling pathways involved in a diseased inflammation state, not seen in resting cells.

Furthermore, we will develop and implement a high-throughput high-content screen for compounds that reverse the LPS-induced phenotype in macrophages as potential drug leads against septic shock. We will screen for small molecule inhibitors of the LPS-induced activation of macrophages. In a preliminary test of this assay, we screened 360 compounds from a library of drugs and inhibitors with annotated MOAs (SelleckChem). RAW264.7 cells were incubated with dimethyl sulfoxide (DMSO) controls and 360 compounds and $\pm$ LPS for a total reaction time of 24 h. We found that compounds BMS-536924, AG-1024, and genistein affect downstream actin rearrangements and appear to reverse the LPS phenotypic to features similar to the DMSO control. Compounds of interest are those that are highly potent (at least in the low- to mid-nanomolar range) and selective, and have desirable pharmacokinetic properties. Further image analysis tools and pipelines using CellProfiler Analyst²⁶ will be developed to detect the reversal of the inflammatory LPS phenotype using supervised machine learning. I will train a classifier to recognize the -LPS phenotype using DMSO-treated field-of-view-level images. Then the trained and annotated model will be used to automatically detect the -LPS phenotypes in the +LPS samples treated with compound. Compounds that show a reversal of the LPS phenotype will be scored as positive (by matching the phenotypes to unstimulated cells) and carried forward for proinflammatory cytokine assays and viability tests.

FUTURE DIRECTIONS

We will screen the unique collection of botanical and marine natural products against the Immuno-CP assay. We anticipate that training the $\pm$ LPS macrophages with the annotated chemical libraries and fitting the natural product extract fingerprints to those clusters will dramatically improve our ability to identify MOAs and potential anti-inflammatory therapeutic leads. Furthermore, extracts that

cluster with known compounds tend to have the similar chemical components or derivatives that give rise to that same phenotype. Therefore, it is possible to reduce rediscovery of known compounds in complex extracts. The presence of a unique CP fingerprint from the natural product extracts could suggest that there are multiple bioactive compounds in a prefraction or there is a unique compound that does not act by any of the commonly encountered MOA. Follow-up studies on natural product compounds of interest will be done to verify their MOA. The natural products that have unique fingerprints will be identified by assay-guided fractionation of the corresponding extracts and deconvolution or structure elucidation of the bioactive component.

We will utilize LPS-stimulated macrophages to capture bioactive compounds that are involved in pathways related to the innate immune response and build a database toward assigning biological functions to natural product extracts. These contributions are significant because they will resolve fundamental technological barriers for the prediction of MOA in complex natural product mixtures and provide new tools to continue the discovery of biologically relevant diverse compounds with unique chemical space. The establishment of a robust high-content macrophage CP pipeline will illuminate the path for the discovery of new and novel anti-inflammatory therapeutics from natural products in an efficient, cost-effective, and high-throughput manner to combat immune-related conditions such as sepsis.

DISCLOSURE STATEMENT

No competing financial interests exist.

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Address correspondence to:

Tannia A. Lau, BS

Department of Chemistry and Biochemistry

University of California Santa Cruz

Santa Cruz, CA 95064

E-mail: talau@ucsc.edu

Abbreviations Used

CP = cytological profiling
LPS = lipopolysaccharide
MOA = mechanism of action