Chemistry & Biology, Vol. 10, 481–486, June, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S1074-5521(03)00127-3

The Platelet as a Model for Chemical Genetics

Minireview

Robert Flaumenhaft* and Derek S. Sim
Center for Hemostasis and Thrombosis Research
Beth Israel Deaconess Medical Center
Harvard Medical School
Boston, Massachusetts 02115

Chemical genetics is an emerging strategy in chemical biology that promises to bring the power of true genetics to mammalian systems and facilitate the transfer of biological discoveries to therapeutics. The platelet is an anucleate cell with several features that render it suitable for chemical genetic analysis. This review addresses the benefits and challenges of chemical genetics using platelets as a model system.

Chemical Genetics

Several relatively recent advances from various fields have converged to create the opportunity to analyze complex biological systems using small molecules. Sizeable small molecule libraries containing drug-like compounds have become commercially available. In addition, advances in diversity-oriented synthetic methods have lead to the creation of small molecule libraries with enhanced diversity and complexity for probing biological systems [1]. The adaptation of commonly used laboratory instruments to high-throughput formats has enabled screening platforms capable of assaying large numbers of compounds for informative phenotypes [2]. In addition, software has been developed that simplifies the analysis of the volumes of data derived from high-throughput assays. These advances have contributed to unprecedented interest and activity in the field of chemical biology.

The use of small molecules to perturb complex biological systems in order to define the roles of specific proteins has been termed chemical genetics based on its analogy to classic genetic approaches [3–5]. For example, reverse genetics (from gene to phenotype) refers to the manipulation of a known gene, creation of a cell or organism harboring the genetic mutation or deletion, and thorough phenotyping of the altered cell or organism. This strategy is used to determine the role of a specific gene in the physiology of a cell or organism. By analogy, reverse chemical genetics is a process whereby the selectivity of a small molecule for a specific molecular target is established, the small molecule is introduced into a complex biological system such as an organism or cell, and the alteration in phenotype is determined [5]. Forward genetics (from phenotype to gene) is a strategy in which complex biological systems are studied by randomly mutating genes, screening for informative phenotypes, and then identifying the gene mutation responsible for the phenotype of interest. By analogy, the term forward chemical genetics has been used to describe a strategy in which small molecules are used much like mutations are used in classical genetics to perturb gene function [5]. Small molecules that impart a desired phenotype in the biological system of interest are selected by high-throughput screening and their molecular targets identified. One advantage of a chemical genetic approach is that once the target of a small molecule is identified and its specificity proven, the molecule serves as a versatile probe that can cause conditional perturbations of diverse biological systems (from enzyme assays to cell cultures to mammalian organisms). Furthermore, unlike a genetic mutation, such a probe has immediate potential as a lead compound for a therapeutic. Thus, the incentive to achieve widely applicable chemical genetic strategies for analyzing varied complex biological systems is great. Yet, there are disadvantages to this approach. Systematic strategies for identifying the targets of active compounds and proving their specificity have not become routine. For these reasons, biological systems must be carefully selected for this approach. This article discusses the platelet as a cellular model for chemical genetic analysis.

Pharmacology of Platelet Activation

Platelets constitute the primary cellular component of hemostasis in mammalian organisms. Their activity must be exquisitely regulated since inadequate activity leads to inappropriate bleeding and excessive activity leads to deleterious thrombosis. Several characteristics of the platelet have rendered it a popular model for studies using small molecules. Perhaps the most important of these is the fact that platelets are anucleate. Thus, genetic manipulation of platelets is difficult. Efforts to study platelets using genetics have been limited largely to the study of platelets derived from transgenically altered murine models and nucleated cells transfected with platelet proteins. A second reason human platelets have been used extensively for pharmacological studies is that, as circulating cells, they are easy to obtain. They do not require cell culture and standard purification techniques are rapid and reproducible. The primary physiologic role of platelets is to survey the integrity of the circulatory system and respond rapidly and robustly at sites of vascular injury. In this capacity, platelets undergo a dramatic phenotypic change in response to a large variety of agonists. Manipulations of this vigorous response by small molecules are readily monitored using routine assay systems such as aggregometry to detect platelet aggregation and luminometry to detect platelet granule release. Another reason why platelets are widely used in pharmacologic studies is their importance in human disease. Platelet-mediated arterial thrombosis, which causes myocardial and cerebral infarction, is the leading cause of morbidity and mortality in the industrialized world. Manipulation of platelet function using small molecules has proven to be an effective means of limiting mortality from acute thrombosis. The fact that platelets are anucleate, are easy to obtain, demonstrate a robust response in biological assays, and

*Correspondence: rflaumen@bidmc.harvard.edu

are an important therapeutic target has made them a popular model for studies using small molecules.

The earliest examples of the use of small molecules to understand platelet physiology were in the discovery of platelet agonists. Adenosine diphosphate (ADP) was purified as a platelet-activating factor from extracts of red blood cells based on its ability to cause platelet clumping [6]. Epinephrine was also found to induce platelet aggregation [7]. Chemists discovered that thromboxane A_2 activated platelets while investigating the metabolism of prostaglandin endoperoxides [8]. Stable thromboxane A_2 mimetics such as U-46619 have since been used to study the role of the eicosanoid pathway in platelet activation. Several compounds that affect signal transduction mechanisms also cause platelet activation. For example, there is an extensive literature using Ca^{2+} ionophores such as A23187 to study the role of $[Ca^{2+}]_i$ flux in platelet activation. Phorbol esters have been used widely to evaluate the role of protein kinase C (PKC) in platelet activation. These pharmacological tools represent only a small fraction of platelet-activating small molecules that have been used to study the process of platelet activation.

Inhibitory small molecules have also been used extensively to elucidate mechanisms of platelet activation. Aspirin has been instrumental in defining the role of cyclooxygenases in platelet activation [9]. Isoform-specific inhibitors of phosphodiesterases (PDE) such as milrinone are also used clinically and have been invaluable in establishing the role of cAMP in platelet function [10]. Small molecule serine/threonine and tyrosine kinase inhibitors, phosphatase inhibitors, lipase inhibitors, calcium chelators, and intracellular protease inhibitors have been used to study platelet signal transduction. Thrombin receptor antagonists such as RWJ-56110 that specifically block protease-activated receptor-1 (PAR-1) have been used to study the role of thrombin-mediated signaling in platelet activation [11]. Thienopyridine derivatives such as clopidogrel are platelet ADP receptor antagonists that are routinely used in the treatment of thrombotic disorders [12]. Thus, inhibitory small molecules have been used both as therapeutics and as molecular probes of platelet function.

Perhaps more than in any other cell, signal transduction in platelets has been defined by small molecules. Yet despite the utility of small molecules in characterizing platelet activation, there are several limitations with this approach. Inhibitory compounds used to define platelet activation are selected based on their activity against known targets. This strategy precludes the discovery of new molecules involved in platelet signaling pathways. In particular, the strategy of using inhibitory compounds with known targets excludes the possibility of identifying novel platelet-specific targets. Such platelet-specific targets are of special interest in the discovery of novel antiplatelet agents. Another disadvantage of the classic pharmacological approach is that it does not easily provide for a systematic comparison of the efficacy of large numbers of inhibitors. Typical studies include only a few inhibitory compounds. Comparisons of the results of different studies are complicated by the fact that assay conditions vary in significant manners. One source of variation is the platelet preparation. Platelets can be drawn into a variety of anticoagulants, assayed in plasma, purified by gel filtration or centrifugation, and resuspended into a variety of buffers. A second source of variation is the assay conditions. Different studies use different concentrations of platelets, different concentrations of agonists and inhibitory compounds, static conditions, agitation, or flow. Since compounds that inhibit platelet activation have not been studied systematically, their activities cannot be directly compared. What is needed to address these issues is a systematic screening strategy of antiplatelet agents that includes active compounds with unknown targets.

A Chemical Genetic Analysis of Platelet Activation

In order to enable the discovery of novel signaling pathways in the platelet and to systematize the pharmacological study of platelet activation, we have used a forward chemical genetic analysis of platelet function. The three most widely studied and readily assayed platelet functions are granule secretion, aggregation, and shape change. All three responses can be elicited by a variety of agonists and, thus, can be used to study platelet activation. We chose to screen for compounds that inhibited agonist-induced platelet secretion since assays of granule secretion most readily lend themselves to a high-throughput format. Our cell-based assay evaluates agonist-induced release of ADP/ATP from platelet granules using a luciferin-luciferase detection system. Blood-banked platelets are used for this assay. The assay demonstrates a signal to noise of 63:1 with a coefficient of variance of 0.14. Approximately 6,000 compounds/day can be screened. To date, we have focused on screening commercially available structurally characterized drug-like compounds of $<$500 Da. The assay has enabled discovery of novel, structurally distinct inhibitors of platelet activation at a rate of about 0.05% compounds screened. This forward chemical genetic analysis of platelet function has lead to the discovery of a large number of structurally distinct, novel inhibitors of platelet activation. The great challenge of a forward chemical genetic analysis of complex biological systems, however, is not discovery of novel active compounds. Rather, the challenge lies in the characterization of the compounds.

Characterization of newly discovered small molecules involves demonstrating the heterogeneity of the compounds, determining whether or not the compounds act selectively, and identifying the molecular targets of the compounds. Establishing heterogeneity of the various compounds discovered by the high-throughput screen is an important means of assessing the screen itself. A high-throughput assay of a complex biological system that identifies compounds that act on a variety of targets enables interrogation of multiple pathways of the system. In contrast, an assay that identifies a group of inhibitors directed at a common target will only yield information about a single aspect of the biological system. Thus, it is important to ascertain the functional heterogeneity of the identified compounds. The value of a small molecule as a molecular probe is directly proportional to its selectivity. Nonselective compounds are of little use in the study of complex biological systems and need to be eliminated as early as possible in the characterization process from further analysis.

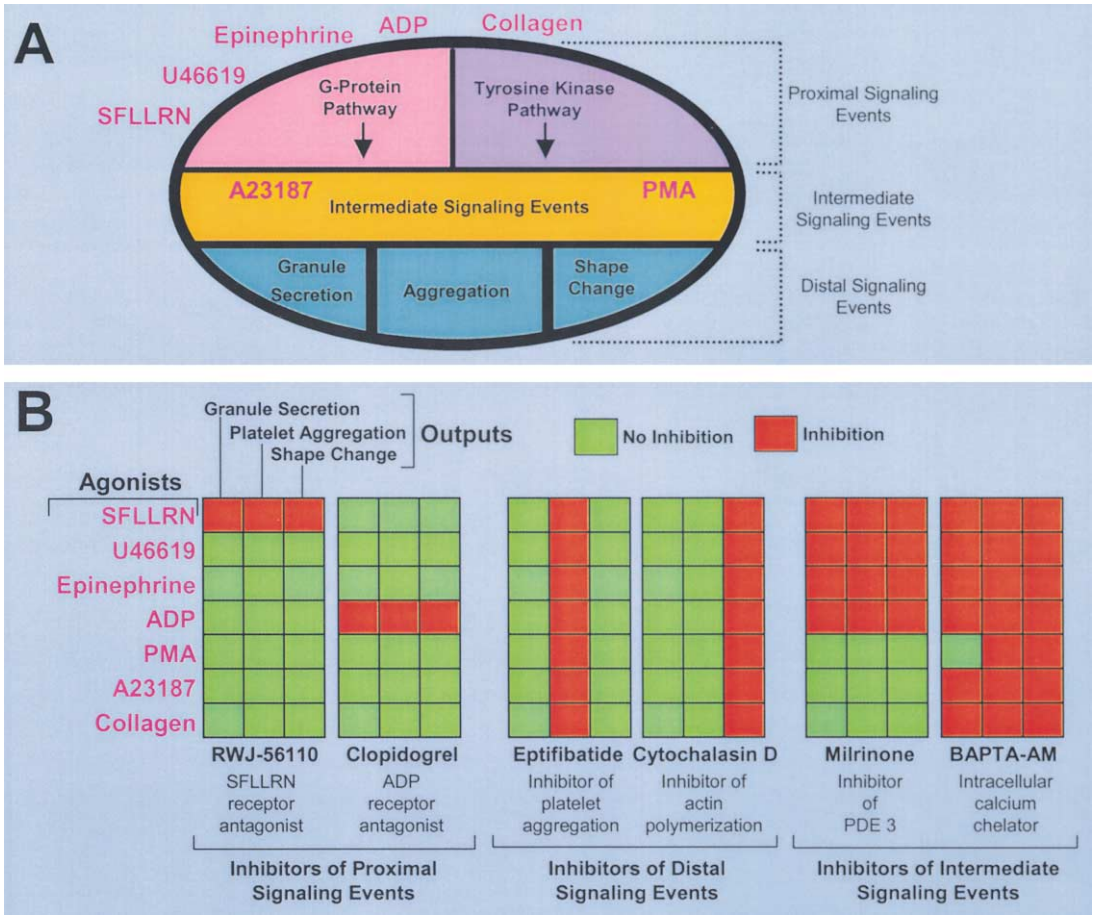

Figure 1. Characterization of Antiplatelet Compounds Using Chemical Phenotyping

(A) Platelet activation can be stimulated by multiple agonists. Some agonists (*SFLLRN*, *U-46619*, *epinephrine*, and *ADP*) act via cell surface receptors that are coupled to G protein-mediated signaling pathways. *Collagen* stimulates platelets via a cell surface receptor that is coupled to tyrosine kinase-mediated pathways. Other platelet agonists (*PMA* and *A23187*) stimulate platelets by stimulating intermediate signaling events. Three platelet functions (*granule secretion*, *aggregation*, and *shape change*) can be rapidly assayed using standard procedures.

(B) An active compound identified by high-throughput screening will inhibit activation induced by different agonists and will inhibit different platelet functions depending on its molecular target. The pattern of inhibition of a particular antiplatelet compound can be used to assess the mechanism by which the compound inhibits platelet function. Several known platelet inhibitors are presented as examples.

Target identification is the component of a forward chemical genetic screen that provides the molecular understanding of a phenotype. Thus, target identification of diverse and highly selective molecular probes is the primary goal of such a screen.

Chemical Phenotyping

In our efforts to characterize antiplatelet compounds identified using our high-throughput screen, we have used an approach termed chemical phenotyping. Chemical phenotyping is a method in which the ability of a small molecule to perturb specific cell functions (outputs) induced by specific agonists (inputs) is determined in standard biological assays. The effect of the small molecule is tested on multiple input-output pairs in order to isolate the activity of the compound to local signaling networks. A local signaling network is defined as the subset of molecules required to produce a specific response following exposure to a particular agonist. Once sufficiently localized signaling networks affected by an antiplatelet agent are identified, a list of candidate targets can be generated and the effect of the small molecule on these candidate molecules can be tested in purified systems.

Chemical phenotyping can assess the heterogeneity, selectivity, and molecular targets of inhibitory compounds identified by high-throughput screening. Systematic comparisons of the inhibitory activity of various compounds in different assays rapidly demonstrate whether they have similar modes of inhibition. In this manner, heterogeneity is readily established even in the absence of target identification. Specificity of compounds is also assessed by this method. For example, if a compound inhibits the activity of two local signaling networks that are known to be unrelated, then the compound probably does not act specificity. Alternatively, the compound may identify a novel crosstalk between two signaling networks that were previously considered independent. Target identification using chemical phenotyping is performed in conjunction with informatics strategies using widely available structure- and litera-

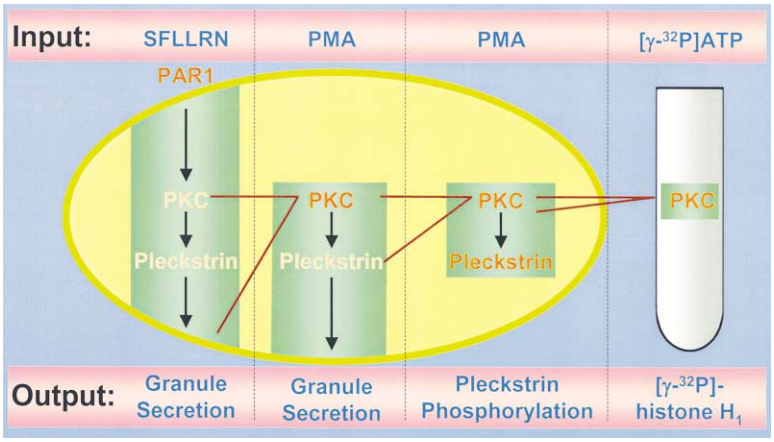

Figure 2. Partitioning Signaling Pathways Using Input-Output Pairs

SFLLRN induces granule secretion via a signaling pathway that includes activation of PKC and pleckstrin phosphorylation. PMA induces platelet granule secretion via activation of PKC. An inhibitor that blocks SFLLRN-induced granule secretion is therefore tested to determine whether or not it is able to inhibit PMA-induced granule secretion. If so, then the inhibitor is acting at or distal to PKC. To determine whether the compound is acting proximally or distally to pleckstrin phosphorylation, the ability of the compound to inhibit PMA-induced pleckstrin phosphorylation is determined. If the compound inhibits PMA-induced pleckstrin phosphorylation in the platelet, it is tested for its activity against purified PKC in a platelet-free assay. Thus, by using systematically designed input-output pairs, increasingly smaller signaling networks can be isolated. Once sufficiently localized signaling networks are determined, the target of the compound can be identified in a purified protein system.

ture-based search engines. By enabling the generation of lists of candidate molecules that can be tested in purified systems, these approaches facilitate the process of target identification.

Chemical Phenotyping of Platelet Activation

Several characteristics render the platelet a good candidate for a chemical phenotyping strategy. One, it can be activated by different agonists that act through distinct cellular receptors (Figure 1A). Two, it has several relatively distinct functional outputs (e.g., granule secretion, aggregation, and shape change). Three, agonist exposure results in robust signals that are easily detected in functional assays. Four, the signaling pathways leading to platelet activation have been described in detail. These characteristics are not unique to platelets and the chemical phenotyping strategy could be applied to a forward chemical genetic analysis of a wide variety of complex biological systems. The platelet serves as an example of this strategy.

Recognizing phenotypic patterns. The initial evaluation of a novel inhibitor of platelet activation by chemical phenotyping uses platelet agonists in order to stimulate readily quantified responses. The proximal signaling events of each agonist are well described. If a small molecule inhibits platelet activation induced by only one of the agonists, then the compound is acting at or near the receptor for that agonist. Furthermore, the compound will inhibit every platelet function induced by that agonist. For example, the thrombin receptor antagonist RWJ-56110 will inhibit activation of all platelet functions induced by the thrombin receptor agonist peptide SFLLRN but will not inhibit activation induced by any other agonists [13]. Thus, the pattern of inhibition of a compound that acts specifically on a proximal receptor will be horizontal in the matrix depicted in Figure 1B. A compound may also act distally in a signaling cascade, inhibiting activation of only one platelet function. For example, cytochalasin D will inhibit platelet shape change without inhibition of platelet granule secretion or platelet aggregation [14]. Cytochalasin D will inhibit shape induced by any agonist. Thus, the pattern of inhibition of compounds that inhibit distal events in platelet signaling will be vertical in the matrix shown in Figure 1B. If the compound inhibits activation induced by all agonists and demonstrates no selectivity for a particular platelet function, then it is most likely a nonspecific inhibitor. By this method, the selectivity and targets of compounds that act very proximally or distally in a signaling pathway can be determined.

A more challenging task is to interpret patterns of inhibition that result from compounds that interrupt intermediate signaling events. The tendency of signaling pathways to diverge, loop, and converge diminishes the likelihood that inhibition by compounds that affect intermediate signaling events will yield simple horizontal or vertical patterns of inhibition. The fact that both the functional activity and targets of many inhibitors of platelet activation have previously been identified, however, enables pattern analysis as a means to characterize the mechanism of an unknown inhibitor. If pattern analysis suggests a specific signaling network, then more refined functional assays can be used to assess the hypothesis. For example, we have identified several compounds that inhibit phosphodiesterases based on the pattern of inhibition compared with well-studied phosphodiesterase inhibitors such as milrinone (Figure 1B). Novel antiplatelet agents that demonstrated the same pattern of inhibitory activity as milrinone were tested for their effect on agonist-induced cAMP levels. These compounds caused increased agonist-induced cAMP levels and were subsequently found to inhibit phosphodiesterase in assays of purified proteins. The availability of purified and/or recombinant proteins and a wide variety of enzyme assays that are offered on a fee for service basis by several commercial vendors render this approach feasible.

Input-Ouput Pairs. When the patterns of inhibition fails to suggest a target, then more detailed assays are required. The goal of these assays is to partition signaling pathways into progressively more localized signaling networks (Figure 2) and determine which of the localized signaling networks is affected by the compound of interest. Just as importantly, these assays will identify aspects of signaling cascades in which the compound has no activity. Assays that are perturbed by the small molecule will provide target information. Assays that are

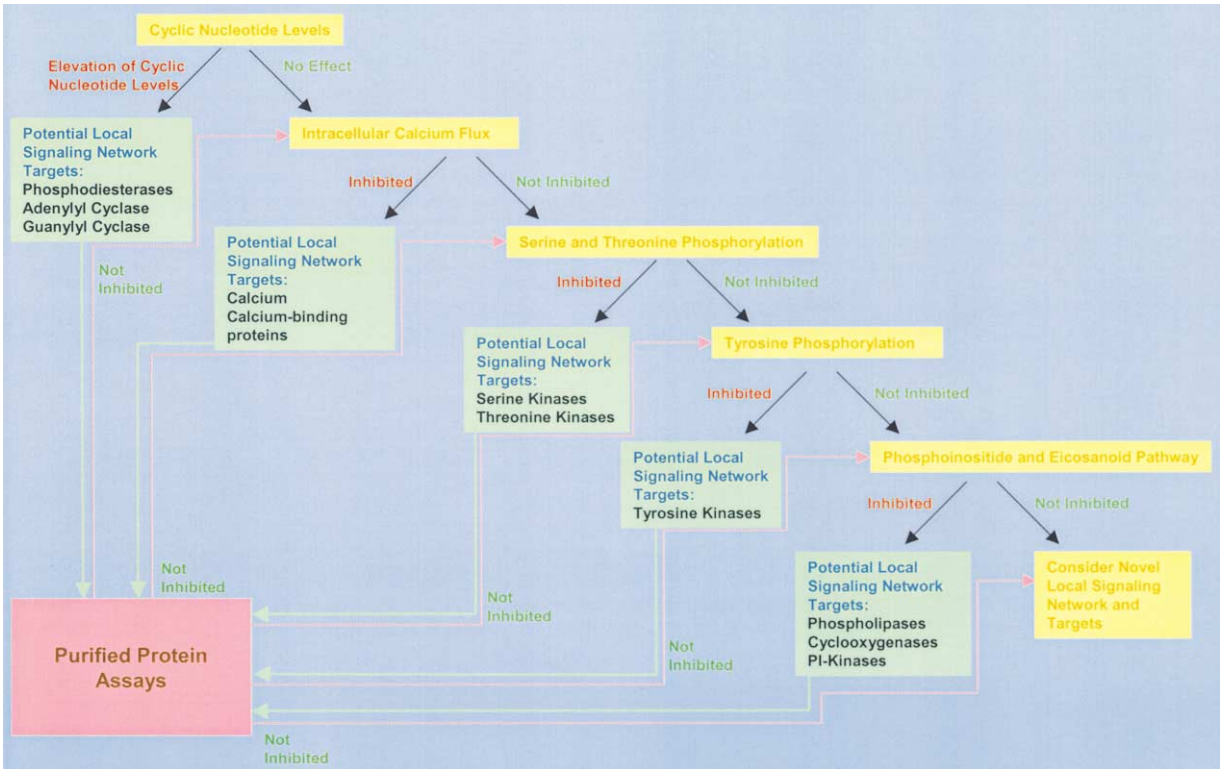

Figure 3. A Generalized Approach to Chemical Phenotyping

Small molecules that inhibit platelet activation induced by specific agonist(s) are tested in a series of assays. The ability of the compound to inhibit platelet functions (yellow boxes) induced by the specific agonist(s) is determined. If the inhibitor does inhibit in the specific assay and a list of candidate targets can be generated based on its activity (categories of candidate molecules are shown in the green boxes), then it is tested against purified target candidates (red box). If the compound shows no activity in the specific assay or it shows no activity against the purified candidate targets, then it is tested in an assay that interrogates a different signaling network. This process is repeated until a local signaling network that includes the candidate target is identified.

not perturbed by the small molecule will provide information regarding specificity. This strategy begins with the identification of an input-output pair that is inhibited by the compound. For example, we have screened for compounds that inhibit granule secretion induced by the thrombin receptor agonist peptide, SFLLRN. Several of the signaling molecules involved in SFLLRN-induced granule secretion have been identified. We can therefore use an agonist that stimulates secretion via a signaling molecule distal to the thrombin receptor, PAR-1, and determine whether our compound also inhibits activation induced by this agonist. For example, phorbol 12-myristate 13-acetate (PMA) stimulates platelet secretion via its interaction with PKC. Inhibition of PMA-induced granule secretion by the small molecule indicates that it inhibits either PKC or signaling molecules distal to PKC involved in granule secretion (Figure 2). In this case, we would refine the input-output pair. For example, we could evaluate whether the small molecule inhibits PMA-induced pleckstrin phosphorylation. If so, then we would determine whether the compound inhibits the activity of purified PKC. If the small molecule fails to inhibit PKC-induced granule release, we would evaluate its ability to inhibit signaling pathways proximal to PKC, such as SFLLRN-induced diacylglycerol production. The approach of identifying progressively smaller local signaling networks requires detailed knowledge of signaling pathways of a particular system. In addition, this approach puts a premium on the availability of agonists that function via specific signaling molecules (i.e., PMA and Ca^{2+} ionophore). If these prerequisites are met, this chemical phenotyping strategy can enable investigators to focus their target identification efforts to a limited number of candidate proteins. This strategy also facilitates identification of compounds that act nonspecifically (i.e., compounds that inhibit multiple unrelated localized signaling networks).

Lack of adequate input-output pairs may preclude identification of the target of a compound or even its localized signaling network. Thus, more general approaches may be required. Our generalized approach involves assaying an active compound for its ability to inhibit agonist-induced second messenger systems and interfere with posttranslational modifications. A schematic of this approach is presented in Figure 3. Initial assays are designed to determine the effects of the inhibitory compound on agonist-induced cAMP and cGMP levels. Cyclic nucleotides are assessed in initial testing because their levels can be assayed using routine methods and their influence on platelet activity is global. Thus, compounds that perturb cyclic nucleotide levels will affect multiple signaling pathways. Compounds that

affect agonist-induced $[Ca^{2+}]_i$ flux also demonstrate global effects on platelet function. Agonist-induced $[Ca^{2+}]_i$ flux in platelets is readily assayed by standard methods. Thus, compounds that do not affect agonist-induced cyclic nucleotide metabolism are analyzed for effects on agonist-induced $[Ca^{2+}]_i$ flux. The most well-characterized posttranslational modification that we screen for is phosphorylation. Immunoblots of platelet proteins separated by two-dimensional electrophoresis probed with anti-phosphoserine/phosphothreonine or anti-phosphotyrosine antibodies provide a global assessment of the effect of the compound on kinase activity. Using mass spectroscopy, it may be possible to identify specific substrates of affected kinases. Such identification will facilitate the generation of a list of candidate proteins. Lipid-derived second messengers play an essential role in platelet activation. If no effects of the inhibitory compound are observed in the assays above or if assays of purified proteins fail to reveal targets, then the effects of the compounds on agonist-induced phospholipid metabolism are evaluated. More specialized studies can be performed in a collaborative basis in laboratories in which these techniques are routine since the small molecules are convenient to transport and use. This strategy can reveal the targets of antiplatelet compounds. More consistently, however, it will identify compounds that act nonspecifically and will characterize signaling networks in which compounds act.

Lessons from Platelets

Recent advances in synthetic chemistry and laboratory automation have brought to individual laboratories the ability to discover and characterize novel small molecules with unique biological properties. We have applied the strategy of using small molecules in a forward chemical genetic screen to study platelet activation. Platelets are anucleate and, thus, are a logical system to study using chemical genetics. Questions that cannot easily be addressed in the platelet by standard molecular biological approaches can be studied using newly discovered and well-characterized molecular probes. Furthermore, because platelets have a dramatic response to agonists, development of straightforward and efficient high-throughput assays capable of discovering novel antiplatelet agents is feasible. The aim of a chemical genetics strategy, however, is not simply to discover compounds that perturb the function of complex systems. It also includes the use of these compounds to define the function of specific molecules in these complex systems. This goal requires that the molecular target of the compounds be identified and that the specificity of the compounds be proven. So what can our experience with the platelet tell us about chemical genetics?

The lesson from the platelet is to focus on phenotype. The strategy of using a series of biological assays to characterize the mechanism of action of an inhibitory compound is not limited to studies of platelets or signal transduction. Any complex biological system that can be divided into smaller input-output pairs is a candidate for such an approach. Indeed, the activity of small molecules in biological assays has been used for decades to facilitate the identification of molecular targets of active compounds. Extensive systematic phenotyping enables rapid identification of compounds that act nonspecifically, thus instructing the elimination of nonspecific molecules from further analysis. This approach also allows identification of compounds with particularly interesting or novel activities. This ability to rapidly prioritize compounds for further evaluation is a significant asset in performing chemical genetic analyses. If the biological system is reasonably well characterized, then this approach will facilitate the generation of lists of candidate compounds in order to identify the compound target. This biological approach to characterization of compounds has limitations. The strategy cannot identify novel targets. It can only characterize novel activities. Once a compound that demonstrates a novel activity is identified, its target will need to be purified by alternative strategies. In addition, proof of specificity is limited to the particular assays and system in which it is studied. Nonetheless, our experience with a chemical genetic analysis of platelet activation demonstrates that carefully designed biological assays can go a long way to realizing the promise of molecular dissection of complex biological systems using small molecules.

Acknowledgments

Robert Flaumenhaft is supported by NIH grant HL63250 and is a Burroughs Wellcome Fund Career Awardee. We thank the Institute of Chemistry and Cell Biology at Harvard Medical School for their assistance in performing high-throughput screening for antiplatelet compounds.

Selected Reading

1. Schreiber, S.L. (2000). Science *287*, 1964–1969.
2. Walling, L.A., Peters, N.R., Horn, E.J., and King, R.W. (2001). J. Cell. Biochem. Suppl. *37*, 7–12.
3. Lokey, R.S. (2003). Curr. Opin. Chem. Biol. *7*, 91–96.
4. Mitchison, T.J. (1994). Chem. Biol. *1*, 3–6.
5. Stockwell, B.R. (2000). Nat. Rev. Genet. *1*, 116–125.
6. Gaarder, A., Jonsen, J., Laland, S., Hellem, A., and Owren, P.A. (1961). Nature *192*, 531–532.
7. O'Brien, J.R. (1963). Nature *200*, 763–764.
8. Hamberg, M., Svensson, J., and Samuelsson, B. (1975). Proc. Natl. Acad. Sci. USA *72*, 2994–2998.
9. Roth, G.J., Stanford, N., and Majerus, P.W. (1975). Proc. Natl. Acad. Sci. USA *72*, 3073–3076.
10. Haslam, R.J., Dickinson, N.T., and Jang, E.K. (1999). Thromb. Haemost. *82*, 412–423.
11. Chackalamannil, S. (2001). Curr. Opin. Drug Discov. Devel. *4*, 417–427.
12. Sharis, P.J., Cannon, C.P., and Loscalzo, J. (1998). Ann. Intern. Med. *129*, 394–405.
13. Andrade-Gordon, P., Maryanoff, B.E., Derian, C.K., Zhang, H.C., Addo, M.F., Darrow, A.L., Eckardt, A.J., Hoekstra, W.J., McComsey, D.F., Oksenberg, D., et al. (1999). Proc. Natl. Acad. Sci. USA *96*, 12257–12262.
14. Addo, J.B., Bray, P.F., Grigoryev, D., Faraday, N., and Goldschmidt-Clermont, P.J. (1995). Arterioscler. Thromb. Vasc. Biol. *15*, 1466–1473.