Discovery-Based Strategies for Studying Platelet Function

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Abstract: The platelet is an anucleate cell, complicating efforts to study platelet function by traditional genetic means. Discovery-based strategies have lead to the identification of pharmacological agents capable of targeting specific proteins critical for platelet activation. This review will address the evolution of discovery-based strategies to identify probes that are at once useful reagents for studying platelet activation and effective therapeutics.

Key Words: Platelets, chemical genetics, bioinformatics.

INTRODUCTION

Platelet activation is a central event in myocardial infarction and stroke. An essential endeavor towards the development of improved anti-platelet therapies has been to understand the mechanisms by which platelets become activated following exposure to physiologic stimuli. This objective has been complicated, however, by the fact that the platelet is anucleate. In nucleated cells, the study of signal transduction has relied heavily upon genetics and molecular biology to identify and map signaling pathways. The strategies of deleting, overexpressing, or modifying specific gene products in cellular and animal models have been among the most widely used and successful approaches in molecular biology. In the anucleate platelet, however, genetic manipulation is substantially more complicated, requiring the use of model cell lines or engineering of genetically modified animals. Platelet biologists have therefore been more reliant on pharmacological manipulation using small molecules to interrogate platelet signaling pathways.

Highly selective small molecules that alter protein function can be used in a manner analogous to that of genetic modifications. Provided a chemical probe is specific for a known target, its activity in platelet function assays can reveal the role of its target in platelet activation. Such chemical probes have several advantages [1]. The use of these small molecules in both platelets and living animals is exceedingly convenient. If the compound is soluble and cell-permeable, it is simply added to the biological system of interest without any manipulations such as transfection of cells or genetic engineering of mice. Small molecules cause conditional alterations of protein function, analogous to conditional mutations, since the investigator determines when they are added to the system. Thus, proteins involved in development can be studied in normal adult organisms. In addition, effects of these probes are titratable, enabling correlations between dose and function. Small molecules can target lipids, carbohydrates, ions, and other biologically important molecules not directly amenable to direct genetic manipulation [1]. Furthermore, understanding the chemical basis of the interaction of an active compound with its target can demonstrate intramolecular mechanisms of protein activity [1]. An additional advantage of these small molecules is that they are sometimes appropriate lead compounds for the development of therapeutics. Disadvantages of this approach involve discovery of active compounds, lack of a systematic strategy for target identification, and the difficulty in establishing the specificity of a compound.

Several strategies have been used to identify compounds capable of targeting proteins involved in platelet activation. Anti-platelet agents have derived from sources ranging from folk remedies found serendipitously (e.g., aspirin) [2] to compounds engineered by rational drug design (e.g., GPIIbIIIa inhibitors such as tirofiban and eptifibatide). High-throughput screening programs have provided an important source of compounds that have served both as useful therapeutics and as chemical probes to enhance our understanding of platelet activation and signaling mechanisms. Classical therapy-oriented screening approaches, screens targeting specific proteins, chemical genetic screens, and screens relying on bioinformatics have all identified anti-platelet agents with utility in studying platelet signal transduction. This review will focus on the evolution of discovery-based approaches to identify compounds that are useful for studying platelet biology and consider structure activity relationships of certain of these compounds.

THERAPY-ORIENTED HIGH-THROUGHPUT STRATEGIES

Thienopyridines

The theinopyridines, including ticlopidine and clopidogrel, are examples of compounds identified using a discovery-based approach that have proven useful both in understanding platelet signaling and as anti-platelet therapies. The first clinically approved theinpyridine was ticlopidine. Ticlopidine was initially discovered in 1972 through *in vivo* screening tests that were directed at identifying new anti-inflammatory compounds [3]. It was subsequently found to be anti-thrombotic in rat models of pulmonary thrombosis [4]. The discovery and early characterization of ticlopidine

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1389-5575/08 $55.00+.00 © 2008 Bentham Science Publishers Ltd.

was performed in animals. Indeed, only metabolites, and not the parent compound itself, possesses the critical anti-platelet effect. Clopidogrel was later identified as an analog with superior anti-platelet activity [5, 6]. Hepatic metabolism via activation by cytochrome P450-1A is required to produce the active metabolite of clopidogrel that effectively inhibits platelet function (Fig. **1**) [7, 8]. This metabolite has been identified, but is highly unstable *in vitro* [9, 10]. An active metabolite of ticlopidine has also been identified [11, 12]. The effects of active metabolites of ticlopidine and clopidogrel on platelet function are irreversible [3]. Recovery of normal platelet function requires production of new platelets.

Fig. (1). Metabolism of clopidogrel. Clopidogrel must be metabolized to attain anti-platelet activity. Metabolism involves conversion of 2-oxo-clopidogrel by cytochrome P450-1A. 2-oxo-clopidogrel is then hydrolyzed to form the active metabolite [8,9]. The active metabolite appears to interact with cysteine 97 on $P2Y_{12}$, thereby interfering with $P2Y_{12}$ homooligomerization [30].

Determination of the target of thienopyridine action has taught us much about the biology of platelet ADP receptors. Thienopyridines were proposed to inhibit ADP-mediated platelet activation based on their ability to inhibit primary ADP-induced aggregation *ex vivo* [13, 14] and on the observation that incubation of platelets with an ADP scavenging system (creatine phosphate/creatine phosphokinase) reproduced the pattern of inhibitory effects observed with thienopyridines [6]. Clopidogrel was subsequently found to inhibit the binding of $[^{32}P]$-2-MeS-ADP to human [15] and rat [16] platelets. Further studies in rat platelets demonstrated two populations of ADP receptors, one that was sensitive to clopidogrel and a second that was resistant [17]. The clopidogrel-resistant ADP receptor was subsequently found to be $P2Y_1$ [18]. The clopidogrel-sensitive ADP receptor was found to be $P2Y_{12}$ [19, 20].

Thienopyridines have been effective probes to analyze the role of ADP-mediated signal transduction in platelet activation and thrombus formation. The observation that clopidogrel only inhibited $P2Y_{12}$- and not $P2Y_1$–mediated signaling contributed to the discovery that both ADP receptors are required for ADP-induced activation of platelets [21, 22]. Clopidogrel has been used as a specific inhibitor of $P2Y_{12}$ in studies demonstrating phosphorylation events downstream of $P2Y_{12}$ [23, 24]. It has been used in experiments indicating a role for $G_{\alpha i}$ signaling in platelet Atk activation [25] and in studies showing a role for $P2Y_{12}$ in protease-activated receptor (PAR)-mediated thromboxane A_2 generation [26]. Clopidogrel has also been useful for demonstrating the role of ADP-mediated platelet activation, and $P2Y_{12}$ specifically, in thrombus formation and embolization [27, 28] [29].

In a more recent study, the active metabolite of clopidogrel was found to reduce homo-oligomerization of $P2Y_{12}$ in HEK cells over-expressing $P2Y_{12}$ [30]. Similarly, orally administered clopidogrel caused disruption of $P2Y_{12}$ oligomerization in rat platelets. Disruption of $P2Y_{12}$ oligomer formation resulted in the partitioning of $P2Y_{12}$ out of lipid rafts, thereby inhibiting signaling through the receptor [30]. Experiments performed with transfectants in which cysteines of the $P2Y_{12}$ receptor were mutated to alanine showed that a C97A mutant was insensitive to the active metabolite of clopidogrel, which failed to reduce $P2Y_{12}$ homo-oligomerization in these cells [30]. This result suggested that cysteine 97 is the target of clopidogrel. With this work both the mechanism of clopidogrel activity and the importance of $P2Y_{12}$ homo-oligomerization in platelet signaling have been elucidated.

The thienopyridines serve as an example of anti-platelet drugs discovered by therapy-oriented screening methods that served as valuable tools in elucidating the role of ADP-mediated signaling in platelet function. The demonstration of their selectivity for $P2Y_{12}$ accelerated the molecular characterization of this receptor and identified $P2Y_{12}$ as a useful target for anti-platelet therapies. Today several new anti-platelet agents that block $P2Y_{12}$ have resulted from target-oriented drug development programs. These include both newer thienopyridines such as prasugrel [31] as well as reversible $P2Y_{12}$ inhibitors cangrelor and AZD6140 [32].

YM-254890

Natural products constitute an important source of compounds for studying cellular function. Discovery-based programs directed at identifying anti-platelet compounds from natural products have yielded important new reagents. YM-254890 is a recent example of such a reagent (Fig. **2**). YM-254890 was discovered in a screening program to find novel inhibitors of ADP-induced platelet aggregation. It was originally identified as an anti-platelet activity in culture broths of *Chromobacterium* sp. QS3666 isolated from soil

YM-254890 (**1**) R=Me
YM-254891 (**2**) R=Et
YM-254892 (**3**) R=CH_2SCH_3

YM-280193 (**4**)

YM-385780 (**5**) R_1=Me, R_2=H
YM-385781 (**6**) R_1=H, R_2=Me

Fig. (2). Structures of YM-254890 analogues and semi-synthetic derivatives (1-6). (Reproduced with permission from Taniguchi et al., Bioorganic and Medicinal Chemistry, 12, 3125. © Elsevier).

[33]. YM-254890 was subsequently purified and its structure determined by NMR [34]. YM-254890 analogs were synthesized to determine critical moieties within the compound [35]. These studies demonstrated that the acyl β-HyLeu-1 residue serves an important role in the inhibitory activity of the compound and that the α,β-unsaturated carbonyl group of the *N*-MeDha is not critical (Fig. **2**). Functional studies in transfected cell lines indicated that YM-254890 inhibited

signaling through $P2Y_1$, but not the other platelet ADP receptors, $P2Y_{12}$ or $P2X_1$ [36]. YM-254890 did not, however, inhibit binding of [^{3}H]-2-MeS-ADP to membranes from $P2Y_1$-expressing cells. Furthermore, YM-254890 inhibited stimulation of cells through other G protein-coupled receptors linked to $G_{\alpha q}$, but not those linked to $G_{\alpha i}$ or $G_{\alpha 15}$ [36]. These data suggested that YM-254890 was a selective inhibitor of $G_{\alpha q}$. This premise was confirmed with the demonstration that YM-254890 inhibited [^{35}S]-GTPγS binding mediated by $P2Y_1$ or M1 muscarinic acetylcholine receptors (which couple to $G_{\alpha q}$) but not $P2Y_{12}$ (which couples to $G_{\alpha i}$) [36]. Thus, YM-254890 is a novel $G_{\alpha q}$-selective inhibitor.

The effect of YM-254890 on platelet function and thrombus formation is consistent with that of a $G_{\alpha q}$ inhibitor. YM-254890 inhibited platelet activation induced via stimulation of $P2Y_1$, PAR1 (which couples primarily to $G_{\alpha q}$ and $G_{\alpha 12/13}$), and U-46619 (which couples to $G_{\alpha q}$) [37]. However, YM-254890 failed to inhibit activation induced by phorbol 12-myristate 13-acetate or calcium ionophores such as A23187 [37]. This inhibitory profile matched the phenotype of the $G_{\alpha q}$-deficient mouse [38] and of a previously identified patient with a $G_{\alpha q}$ deficiency [39]. *In vitro* studies using platelets flowing at 1500 s^{-1} in a perfusion chamber demonstrated that YM-254890 inhibited thrombus formation on collagen under conditions of high shear [37]. *In vivo* studies demonstrated that YM-254890 prolonged occlusion times in both a $FeCl_3$-induced mouse model [40] and in an electrically-induced rat model of thrombus formation in the carotid artery [41]. The compound also shortened time to reperfusion following administration of tissue plasminogen activator in the rat model [41]. Experiments performed in the femoral artery of cynomolgus monkeys demonstrated that YM-254890 inhibited thrombosis formed following ligation-induced stenosis and crush injury [39]. YM-254890 also inhibited neointimal formation following $FeCl_3$ injury and improved blood flow in a rat model of laurate-induced peripheral artery disease [40]. Effects on systemic blood pressure at higher doses, however, were observed and may limit its therapeutic use [40].

Since its discovery and characterization, YM-254890 has become an increasingly popular probe for studying the role of $G_{\alpha q}$ in signaling pathways in a variety of cell systems. YM-254890 was used to evaluate the relative contributions of $G_{\alpha q}$ and $G_{\alpha 12/13}$ in PAR-induced Akt phosphorylation in platelets, demonstrating that $G_{\alpha q}$ does not have a direct effect on Akt phosphorylation [42]. The roles of $G_{\alpha q}$ and $G_{\alpha 12/13}$ in RhoA activation following following exposure to Pasteurella multocida toxin has been evaluated using YM-254890 [43]. YM-254890 has also been used to demonstrate the coupling of newly characterized GPCRs to $G_{\alpha q}$ [44-47]. Thus, despite its potential problems as a therapeutic, YM-254890 has become a useful reagent for studying $G_{\alpha q}$-mediated signal transduction.

TARGET-DIRECTED SCREENS

Target-directed approaches in which a protein of established importance in platelet function is targeted using antibodies (e.g., abciximab targeting of GPIIbIIIa) or rational drug design (e.g., eptifibatide and tirofiban targeting of GPIIbIIIa) have been successfully used for the development of anti-platelet compounds. High-throughput screening of small molecules has also been used to identify inhibitors of specific proteins that mediate platelet activation. One example of such a target is the thrombin receptor, protease-activated receptor 1 (PAR1). PAR1 is an unusual receptor insomuch as it is activated by a tethered ligand generated by cleavage of its N-terminal portion [48]. Several programs within the biopharmaceutical sector have been initiated to develop inhibitors of PAR1. Such programs have included rationale synthetic approaches to create antagonists derived from modifications of the peptide sequence known to activate PAR1 [49, 50], to develop inhibitory peptidomimetics [51], and to identify non-peptide, small molecule antagonists by target-directed screens [52, 53].

One program developed at Schering-Plough Research Institute to identify small molecule inhibitors of PAR1 is particularly illustrative of the target-directed screening approach. Like many target-based high-throughput screens, this program derived from an assay designed to evaluate the binding of a ligand to its cell surface receptor. However, the affinity of the natural peptide ligand for its receptor is relatively low and this interaction is not well suited as the basis for a high-throughput screening assay. Such an assay, therefore, required the development of a potent thrombin receptor ligand termed high-affinity Thrombin Receptor Activating Peptide (haTRAP), initially developed by investigators at Merck Research Laboratories [54]. Human platelets were found to activate in response to haTRAP at concentrations that were 1000-fold less than the required concentration of the natural peptide ligand. This level of potency enabled the development of a high-throughput assay capable of detecting binding of [^{3}H]-haTRAP to human platelet membranes [55]. Several groups of PAR1 inhibitors have been identified using this assay. The first group of low molecular weight PAR1 antagonists to be identified were the pyrroloquinazolines [56]. SCH-79797 and SCH-203099 are pyrroloquinazolines that were found to inhibit binding of [^{3}H]-haTRAP to human platelet membranes with IC_{50}s of 70 nM and 45 nM, respectively [57]. These compounds inhibited PAR1 peptide-induced platelet aggregation with IC_{50}s of 300 nM and 150 nM, respectively, and inhibited α-thrombin-induced platelet aggregation with IC_{50}s of 3000 nM and 700 nM, respectively [57]. Thus, the compounds were approximately 15-40-fold less potent inhibitors of α-thrombin-induced than haTRAP-induced platelet aggregation. In addition, inhibition of α-thrombin-induced activation was transient and less substantial at higher α-thrombin concentrations. The fact that these compounds were much more effective against the agonist used for screening than the physiologic agonist is a common problem of target-based screens. In this case, the fact that α-thrombin activates PAR4, which is also capable of activating platelets [58], likely contributes to the discrepancy between the haTRAP and α-thrombin data. An additional limitation of SCH-79797 is that at higher concentrations, the compound actually behaves as an agonist [59], another potential complication of small molecule inhibitors targeted at ligand binding pockets. Although these properties limit the use of the pyrroloquinazoline derivatives as therapeutic agents, these compounds have been used in research settings as selective antagonists to establish the role of PAR1 in a variety of biological systems including platelets [60-62].

The two other groups of compounds identified as PAR1 inhibitors using the [^{3}H]-haTRAP binding high-throughput assay are benzimidazole derivatives and himbacine derivatives. The most potent benzimidazole derivative inhibited binding of [^{3}H]-haTRAP to platelet membranes with an IC_{50} of 33 nM, inhibited PAR1-peptide-induced platelet aggregation with an IC_{50} of 575 nM, and inhibited α-thrombin-induced platelet aggregation with an IC_{50} of 1500 nM [53, 63]. A himbacine derivative termed SCH-205831 is the most potent small molecule inhibitor of PAR1 identified to date. It was found to inhibit [^{3}H]-haTRAP binding to platelet membranes with an IC_{50} of 11-24 nM and inhibit α-thrombin-induced platelet aggregation with an IC_{50} of 44 nM, despite being inactive against PAR2 and PAR4 [64]. Pharmacokinetic studies of SCH-205831 demonstrated an oral availability of 30% in rats and 50% in cynomolgus monkeys after a dose of 10 mg/kg SCH 205831 [64, 65]. Inhibition of thrombin-induced platelet aggregation was observed in *ex vivo* studies performed with platelets isolated from SCH 205831-treated rats. However, since SCH-205831 was found to induce CYP-2B in rats, metabolites were identified, synthesized, and characterized in hope of finding a more suitable drug candidate [65]. A 7-α-hydroxy metabolite inhibited [^{3}H]-haTRAP binding to platelet membranes with an IC_{50} of 17 nM. This metabolite showed 92% absorption and 89% bioavailability in cynomolgus monkeys [65]. In *ex vivo* studies performed after a 1 mg/kg dose, it showed complete inhibition of haTRAP-induced platelet aggregation at 6 hours and 60% inhibition at 24 hours. This SCH-205831 metabolite remains a viable candidate for an orally available, non-peptidic PAR1 inhibitor.

CHEMICAL GENETICS

While discovery-based approaches have been used for decades in the biopharmaceutical industry, the use of high-throughput screening of small molecules in academic centers is a relatively recent trend. A federal mandate to enhance the translation of knowledge gained by basic research into novel therapeutics has lead to initiatives that support the use of small molecules to study disease-oriented biology in the public sector [1]. The Molecular Libraries Roadmap, a component of the NIH New Pathways to Discovery, includes initiatives to develop novel high-throughput screening assays and libraries. Concurrently, substantial 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 [66]. 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 [67]. 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 and high-throughput screening of chemical libraries in academia [1].

Chemical genetics has developed from this trend as a discipline focused on studying complex biological systems using small molecules identified by high-throughput screening. The term is derived from its conceptual similarity to classical genetic screening [1]. In a classic forward genetic screen, a cell or organism is exposed to a mutagen and screened for a mutant demonstrating a phenotype of interest. The mutant is then evaluated to identify the genetic defect and protein modification that resulted in the selected phenotype. In a forward chemical genetic screen, small molecules are used as the ‘mutagen’ and small molecules that cause a desired phenotype are selected [68]. The protein target of the small molecule is then identified. An advantage of this approach compared to target-based strategies is that entire signaling pathways can be interrogated in an unbiased manner.

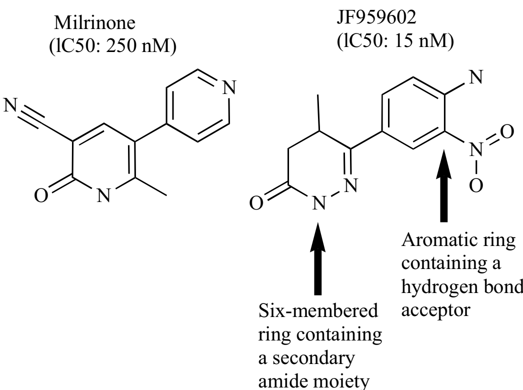

Fig. (3). A chemical genetic screen identified a novel phosphodiesterase 3A inhibitor with similar, but more potent activity compared to established phosphodiesterase inhibitors.

A forward chemical genetic screen of platelet activation performed in collaboration with the Institute of Chemistry and Cell Biology at Harvard Medical School provided a global analysis of platelet signaling [69]. The primary high-throughput screen selected for compounds that inhibited PAR1 peptide-induced release of ADP/ATP from platelet granules using a luciferin-luciferase detection system [69]. The assay demonstrated a signal to noise of 63:1 with a coefficient of variance of 0.14 [70]. A commercially available library of structurally characterized drug-like compounds of <500 Da was screened. Approximately, 6,000 compounds per day could be analyzed. The assay identified eight novel, structurally distinct inhibitors of platelet activation out of over 16,000 compounds, yielding a ‘hit’ rate of about 0.05% compounds screened [70]. Three of the eight compounds identified were inhibitors of phosphodiesterase, indicating that phosphodiesterases have an important influence on PAR1 signaling and/or that these enzymes represent readily drugable targets in the platelet. One of these compounds, termed JF959602, was found to specifically inhibit phosphodiesterase 3A with an IC_{50} of 15 nM [69]. This compound failed to inhibit phosphodiesterase 2 or 5, the other phosphodiesterases in platelets, even at concentrations several hundred-fold greater than those required to inhibit phosphodiesterase 3A. While a novel phosphodisesterase inhibitor, JF959602 possesses a secondary amide moiety in a six-ringed structure that is present in other phosphodiesterase 3A inhibitors and is thought to interact with residues that bind the cyclic phosphate of cAMP (Fig. **3**). JF959602 also possesses an aromatic ring that is coplanar and contains a hydrogen bond acceptor. This compound was subsequently

tested in a murine model of arterial thrombus formation [69]. It inhibited thrombus formation following laser-induced vascular injury with an IC_{50} of approximately 0.25 mg/kg. JF959602 was then used to demonstrate that cAMP levels in circulating platelets control attachment to and detachment from sites of arteriolar injury [69]. Other compounds identified using this screen are being characterized and small molecule high-throughput screens at other academic centers are being pursued to study various components of platelet function.

BIOINFORMATICS

Global analyses of platelets has produced a wealth of information that can direct discovery-based programs. Thousands of platelet proteins have been identified using proteomics [71] or profiling techniques [72]. This information has directed the construction of bioactive peptides for screening in platelets [73]. From a set of 2,900 platelet proteins, Edwards et al. identified 47 highly expressed candidate transmembrane proteins [73]. From among these proteins, they constructed 78 cytoplasmic decamers from conserved sequences found within 30 amino acids from the transmembrane domain. Paralogous peptides (peptides from the same region of related proteins that differed in at least one conserved residue) were also used. Peptides were synthesized with an N-terminal palmitate to enable membrane permeability. The peptides were subsequently screened for the ability to stimulate or inhibit platelet aggregation and secretion. Of the 78 peptides that were screened, 22 were found to be active at 50 µM and 13 were found to be active at 10 µM. One palmitoylated peptide based on the cytoplasmic domain of the immunoglobulin-like CD226 demonstrated an IC_{50} <10 µM. Functional analyses demonstrated that this palmitoylated peptide regulated inside-out but not outside-in integrin activation [73]. Pure computational approaches to identify functional motifs among the peptides failed to recognize most of the active motifs identified using this systematic oligopeptide screening approach. Thus, the high-throughput screening component of this approach was required. These results demonstrate the utility of applying bioinformatics to direct discovery-based peptide screens for identifying biologically important signaling motifs in platelet proteins.

CONCLUSIONS

Perhaps more than in any other cell, the biology of the platelet has been defined by small molecules. Conversely, the platelet continues to be an important model for discovery and characterization of bioactive compounds. Initial efforts to identify anti-platelet compounds using screening techniques were performed within the biopharmaceutical sector and focused on discovery of therapeutic agents. Identification of important biological probes for studying platelet function was more or less a by-product of this endeavor. As more was learnt about essential molecular targets in the platelet, screening programs directed at identifying target-selective compounds developed and have provided a source of useful compounds for evaluating platelet biology (Table **1**). More recently, global approaches such as chemical genetics and bioinformatic discovery are being used to develop novel small molecule probes of platelet function. An advantage of these newer approaches is that since they are not necessarily derived from known pathways (unlike target-directed screens), they have the ability to identify poorly studied or previously unidentified pathways. A disadvantage is that systematic approaches to identify the targets of newly discovered active small molecules have not developed and target identification remains a significant challenge. Similarly, establishing the selectivity of such compounds remains problematic. Development of strategies to streamline and systematize target identification [74-77] and to rigorously evaluate the specificity of small molecules [78] are being developed and may represent the next phase in the evolution of the use of small molecules to study platelet biology.

ACKNOWLEDGEMENTS

R.F. is supported by NIH grant HL63250 and is a recipient of a Grant-In-Aid from the American Heart Association and a Special Project Award from Bayer Healthcare.

Table 1. Compounds, Structures, Targets and Methods of Discovery

Compound	Clopidogrel	YM-254890	SCH205831	JF959602	CD226 peptide
Structure:	H, $COOCH_3$, N, S, Cl	O, HN, O, O, O, O, N, O, O, O, N, O, O, NH, O, OH, O, NH, O, N, O	O, H, H, O, Me, H, H, N, CF_3	N, N, O, O, N, N	*Pal*-RRERRDLFTE
Target:	P2Y12	Gαq	PAR1	PDE3A	CD226
Method of Discovery:	Therapy-oriented screen	Therapy-oriented screen of natural products	Target-directed screen	Chemical genetic screen	Bioinformatics-directed screen

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Received: 06 April, 2007 **Revised: 27 August, 2007** **Accepted: 27 August, 2007**

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