Review of Pilot Phase Probes Produced by the UNMCMD

University of New Mexico, Albuquerque, NM
Larry A. Sklar, Bruce S. Edwards, Eric R. Prossnitz, Chetana M. Revankar, Megan Dennis, Susan M. Young, Juan J. Strouse, Mark B. Carter, Anna Waller, Mark Haynes, Virginia Salas,
Tudor I. Oprea, Cristian G. Bologa, Dan Fara, Andrei Leitao, Ramona Curpan,
Hattie Gresham, Todd Thompson

New Mexico State University, Las Cruces, NM
Jeffrey B. Arterburn, Bj K. Bryant, Chinnasamy Ramesh, Ritwik Burai,

ChemDiv, San Diego, CA
A. S. Kiselyov, M. A. Parker, S. E. Tkachenko, N. P. Savchuck

Introduction

During the Pilot Phase of the Molecular Libraries Program we conducted primary screens on 30 targets, successfully transitioning our flow cytometry screening technology from 96 to 384 well HTS before implementing the first screen. We collected and uploaded more than 4 million data points for PubChem and completed work on 8 novel probes [1-8]. Five of the probes were generated in target-based screens directed at identifying small molecule ligands for specific G-protein coupled receptors [1-5]. The others were generated in cell-based phenotypic screens that were designed to identify small molecules promoting targeted phenotypic responses [6-8].

Target-Based Screens

Small molecule probe development projects incorporating virtual screening, cheminformatics and synthetic chemistry focused on two distinct families of GPCRs have yielded valuable new chemical probes. In each case the screening application required the development of an appropriate small molecule fluorescent probe, and the flow cytometry platform provided inherently biological rich assays that facilitated the identification and optimization of novel antagonists.

FPR family: FPR and FPRL1 Antagonist Probes

The G-protein coupled formylpeptide receptor (FPR) was one of the originating members of the chemoattractant receptor superfamily. FPR promote trafficking of phagocytic myeloid cells to sites of infection and tissue damage where they exert anti-bacterial effector functions and clear cell debris. FPR have also been proposed as prospective targets for therapeutic intervention against malignant gliomas. Natural FPR agonists include bacterial or mitochondrial proteins and the glucocorticoid-regulated protein, annexin I (lipocortin I). FPR-like 1 (FPRL1) shares 69% identity at the amino acid level with FPR and is, like FPR, a seven-transmembrane, G-protein-coupled receptor (GPCR). Identification of a variety of host-derived FPRL1 agonists of pathophysiological provenance supports the concept that cells responding to FPRL1 ligands may contribute to the inflammatory pathology observed in the diseased tissues. FPRL1 agonists have also been reported to elevate leukocyte expression of endogenous tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). FPR is expressed by neutrophils, monocytes, hepatocytes, immature dendritic cells, astrocytes, microglial cells, and the tunica media of coronary arteries. FPRL1 is expressed by phagocytic leukocytes, hepatocytes, epithelial cells, T lymphocytes, neuroblastoma cells, astrocytoma cells, and microvascular endothelial cells. Normal human lung and skin fibroblasts express both receptors. The diverse tissue expression of these receptors suggests the possibility of as yet unappreciated complexity in the innate immune response and perhaps other unidentified functions for the receptor family.

The conceptual approach for a screening assay to identify novel small molecule receptor ligands focused on achieving the simultaneous measurement of binding interactions with both FPR and FPRL1 receptors. This strategy had the potential to improve overall screening efficiency, an advantage of multiplexing, and generated data that reflected receptor specificity as an inherent feature of the screen. A key element of this approach was our development of a fluorescent probe that binds both FPR and FPRL1 with low nM affinity so as to simultaneously report ligand-binding interactions of both receptors in a single assay [9]. Cells expressing human FPR or FPRL1 were fluorescently color-coded to allow their discrimination in flow cytometric analysis of fluorescent ligand

competition. The homogeneous assay format required only ~2 µl to be aspirated from each well and ~11 min for flow cytometry processing of each 384-well plate. This probe development project identified novel ligands for FPR (3570-0208**,** K_i = 95 ± 10 nM) and FPRL1 (BB-V-115**,** K_i = 270 ± 51 nM) [10]. Each was a selective antagonist in intracellular calcium response assays (inactive against the off-target receptor at up to 100 µM agonist concentrations) and the most potent small molecule antagonist reported for its respective receptor to date [10].

Publications resulting from FPR and FPRL1 probes.

Young, S.M.; Bologa, C.M.; Fara, D.; Bryant, B.K.; Strouse, J.J.; Arterburn, J.B.; Ye, R.D.; Oprea, T.I.; Prossnitz, E.R.; Sklar, L.A.; Edwards, B.S. Duplex high-throughput flow cytometry screen identifies two novel formylpeptide receptor family probes. *Cytometry A*, **2009**, *75*, 253-263.

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Oprea, T.I.; Bologa, C.G.; Boyer, S.; Curpan, R.F.; Glen, R.C.; Hopkins, A.L.; Lipinski, C.A.; Marshall, G.R.; Martin, Y.C.; Ostopovici-Halip, L.; Rishton, G.; Ursu, O.; Vaz, R.J.; Waller, C.; Waldmann, H.; Sklar, L.A. A crowdsourcing evaluation of the NIH chemical probes. *Nat. Chem. Biol.,* **2009***, 5*, 441-447.

GPER: Agonist and Antagonist Probes

The G protein-coupled estrogen receptor (GPER), originally designated as GPR30, has emerged as an intriguing signaling molecule enmeshed in the complex pathways through which estrogens regulate diverse physiological processes. Distinguishing the biological roles of GPER -mediated signaling from the classical estrogen receptors, ERα/β, is essential for understanding the fundamental mechanisms of estrogen action. This opportunity required the discovery of new ligands with high selectivity for GPER, and the capacity to specifically activate or inhibit receptor-mediated signaling. The observed cross-reactivity of estrogen and estrogen-derived probes for both classes of estrogen receptors provided a common scaffold that we exploited for a ligand-based virtual screening approach to identify estrogen-like structures of interest for biological screening. A combination of 2D and 3D similarity approaches were used for virtual screening of a library of 10,000 molecules that was constructed using concept of GPCR-privileged substructures [11]. The combined similarity score attributed 40% weight to 2D fingerprints, 40% to the shape-based similarity and 20% to pharmacophore-based similarity to rank the top 100 molecules selected by composite score for biomolecular screening.

We developed a fluorescent estrogen probe, E2-Alexa633 [12], and used it as the basis of a competitive ligand binding assay that could be analyzed by high throughput flow cytometry. A GPER-GFP fusion protein was expressed and cells were gated for high levels of green fluorescence, correlating with GPER expression, in order to maximize the bound fluorescent signal of E2-Alexa633. Cells were permeabilized with saponin to permit access of the charged E2-Alexa633 to GPER. Biomolecular screening of the 100 molecules that had been pre-selected by virtual screening identified G-1, a high-affinity probe for GPER (K_i = 11 nM) [13]. G-1 was a potent agonist for GPER (EC_{50} = 2 nM) but not for ERα/β in intracellular calcium response assays. This probe has subsequently been used to examine the cellular and physiological actions of GPER in a variety of other published studies. Cellular effects of G-1 include activation of calcium fluxes in LHRH neurons and hypothalamic neurons [14,15], spinal neuron depolarization [16], protein kinase Cε activation [17], gene expression [18,19], proliferation [18,20], oocyte meitotic arrest [21], and primordial follicle formation [22]. The role of GPER *in vivo* has been investigated using G-

1 with reported effects that include estrogen-induced thymic atrophy [23], experimental autoimmune encephalomyelitis [24], and vascular regulation [25]. G-1-mediated effects were demonstrated to be absent in GPER knockout mice , establishing the selectivity of this compound for GPER. In one report G-1 did not show any estrogenic effects *in vivo* using the classical estrogen target organs, the uterus and the mammary gland [26]. However, recent studies have revealed G-1 causes a moderate increase in uterine epithelial cell proliferation [27].

Hypothesizing that the hydrogen bond acceptor properties of the ketone group in G-1 were associated with the observed agonism associated with GPR30 binding, and seeking a corresponding structural trigger to block GPR30-mediated signaling, we employed parallel virtual screening and synthetic programs to identify new compounds with altered activation profiles. A substructure search of 144,457 molecules in the MLSMR, performed using a custom JAVA program built using the OpenEye OEJava toolkit, resulted in a focused library of 57 MLSMR molecules. Screening of these compounds for the ability to block estrogen-activated calcium mobilization in GPR30-expressing SKBr3cells identified G15, an antagonist that binds to GPER with an affinity of ~ 20 nM [27]. Evaluation of G15 in cell based and physiologically relevant *in vivo* models of estrogen action suggested that GPER contributes to the proliferative response in the uterus but not towards imbibition, which appears to be mediated exclusively by ERα, and implicated a putative neurological role for GPER in the regulation of depression that may ultimately provide a viable target for the development of new antidepressants [27]. A number of additional studies have been recently published that document use of one or both of these GPER probes [28-83].

Publications resulting from GPER agonist and antagonist probes (selected from a total of 74)

Bologa, C.G.; Revankar, C.M.; Young, S.M.; Edwards, B.S.; Arterburn, J.B.; Kiselyov, A.S.; Parker, M.A.; Tkachenko, S.E.; Savchuck, N.P.; Sklar, L.A;, Oprea, T.I.; Prossnitz, E.R. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat. Chem. Biol.,* **2006**, *2*, 207-212.

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Phenotypic Response Screens

Prostate Cancer

Prostate cancer is a leading cause of death among men due to the limited number of treatment strategies available for advanced disease. Discovery of effective chemotherapeutics involves the identification of agents that inhibit cancer cell growth. Increases in intracellular granularity have been observed during physiological processes that include senescence, apoptosis, and autophagy, making this phenotypic change a useful marker for identifying small molecules that induce cellular growth arrest or death. In this regard, epithelial-derived cancer cell lines appear uniquely susceptible to increased intracellular granularity following exposure to chemotherapeutics. We established a novel flow cytometry approach that detects increases in side light scatter in response to morphological changes associated with intracellular granularity in the androgen-sensitive LNCaP and androgen-independent PC3 human prostate cancer cell lines [84]. Using the HyperCyt high throughput flow cytometry screening platform we identified a piperidine carboxamide substituted aryl-oxazole (**3240581**) that consistently induced increased intracellular granularity in LNCaP cells after four days in culture [85]. Counter-screen analysis with the non-androgen responsive PC-3 prostate cancer cell line suggested that **3240581** affects granularity through an androgen-independent mechanism. Dose response analysis of **3240581** indicated an EC_{50} range of 1–6 microM where LNCaP cells were consistently more affected than PC-3 cells. Microscopic analysis confirmed that **3240581** induced vesicle accumulation in both PC-3 and LNCaP cells [85].

Publications resulting from prostate cancer probe

Haynes, M.K.; Strouse, J.J.; Waller, A.; Leitao, A.; Curpan, R.F.; Bologa, C.; Oprea, T.I.; Prossnitz, E.R.; Edwards, B.S.; Sklar, L.A.; Thompson, T.A. Detection of intracellular granularity induction in prostate cancer cell lines by small molecules using the HyperCyt high-throughput flow cytometry system. *J. Biomol. Screen.,* **2009**, *14*, 596-609.

Dennis, M.K.; Bowles, H.J.; MacKenzie, D.A.; Burchiel, S.W.; Edwards, B.S.; Sklar, L.A.; Prossnitz, E.R.; Thompson ,T. A. A multifunctional androgen receptor screening assay using the high-throughput Hypercyt flow cytometry system. *Cytometry A*, **2008**, *73*, 390-399.

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Oprea, T.I.; Bologa, C.G.; Boyer, S.; Curpan, R.F.; Glen, R.C.; Hopkins, A.L.; Lipinski, C.A.; Marshall, G.R.; Martin, Y.C.; Ostopovici-Halip, L.; Rishton, G.; Ursu, O.; Vaz, R.J.; Waller, C.; Waldmann, H.; Sklar, L.A. A crowdsourcing evaluation of the NIH chemical probes. *Nat. Chem .Biol*., **2009**, *5*, 441-447.

Quorum Sensing

Quorum sensing is a cell-to-cell communication system that permits members of a bacterial population to coordinate their behavior dependent on cell density. The mediators of this communication system are small, diffusible pheromones or autoinducers that are secreted by the bacteria and that accumulate extracellularly. At the appropriate concentration threshold that reflects a sufficient number or quorum of bacteria, the autoinducers signal gene expression programs that direct the coordinated switch of the population to a virulence-associated phenotype. A cell-based assay was used for flow cytometry screening in which the RNAIII promoter, activated in the late stages of the pathway, was fused to a GFP expression vector. An active test compound was one that prevented GFP expression in the presence of a pathway-activating peptide, AIP. This probe development project identified two small molecules with distinctive modes of action. Benzbramarone inhibited AIP-induced production of RNAIII transcripts and of virulence factors α-hemolysin and lipase (IC_{50} = 100-200 nM). Receptor binding studies with FITC-AIP indicated that the primary mechanism of benzbromarone was to inhibit the binding of AIP to the AgrC cellular receptor, the initial step in the bacterial quorum sensing pathway. Inhibitory effects of benzbromarone were elicited in the S. *aureus* RN6390 strain as well as in NM300, a local isolate that belongs to the community acquired methicillin resistant *S. aureus* clone USA300. The second probe, C094-0010 (IC_{50} = 100-200 nM), inhibited AIP-induced virulence factor production in an *S. aureus* strain expressing the Agr3 variant of the accessory gene regulator (*agr*) locus, but had little or no effect on an Agr1 variant strain. Its inhibitory effects were thus selective for AIP signaling associated with the Agr3 genotype. C094-0010 inhibited an element in the AIP signaling pathway downstream of AIP binding to cellular receptors. The targeted pathway element(s) remain to be determined. **C094-0010** has been recommended as a probe of AIP-induced signaling pathways in strains of *S. aureus* expressing the Agr3 variant of the *agr* locus. Neither probe affected bacterial viability.

Publications resulting from quorum sensing probes

Sklar, L.A.; Edwards, B.S. HTS flow cytometry, small molecule discovery, and the NIH Molecular Libraries Initative. In: *Flow Cytometry in Drug Discovery and Development*; Litvin and Marder, Eds; John Wiley & Sons: Hoboken, NJ, 2010; . pp 71-98.

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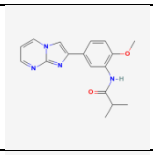
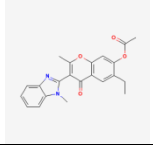
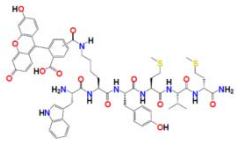
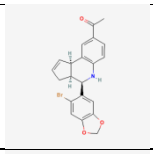
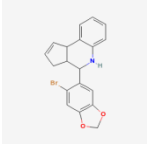
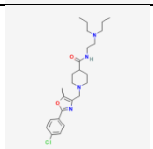
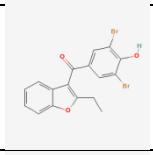
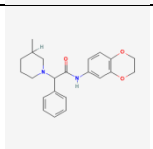
Table Summarizing Pilot Phase Probes

Structure	ML#	CID	Target
	ML048	3092570	FPR
	ML047	6622773	FPRL1
	ML049	-1000	FPR/FPRL1
	ML051	5322399	GPER
	ML050	3136844	GPER
	ML052	3240581	Prostate Cancer
	ML054	2333	Quorum Sensing
	ML053	3240990	Quorum Sensing

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