VAV1 INHIBITION:

EXPLORING DRUG DESIGN STRATEGIES FOR TARGETING GEF ACTIVITY

Ph.D. THESIS

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El **Dr. XOSÉ RAMÓN GARCÍA BUSTELO**, Profesor de Investigación del Consejo Superior de Investigaciones Científicas,

CERTIFICA

Que el trabajo de tesis titulado **"VAV1 inhibition: exploring drug design strategies for targeting GEF activity"**, presentado por **Dª ISABEL DE ROJAS DE PABLO** para optar al Grado de Doctor por la Universidad de Salamanca, ha sido realizado bajo mi dirección en el Centro de Investigación del Cáncer de Salamanca (USAL/CSIC). Considerando que cumple con las condiciones necesarias, autorizo su presentación a fin de que pueda ser defendido ante el tribunal correspondiente.

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ABSTRACT

VAV1 is a RHO guanine nucleotide exchange factor (GEF) predominantly expressed in hematopoietic cells, where it activates RHO GTPases —primarily RAC1—and plays essential roles in T cell development, immune response and cell signaling. Recent studies have elucidated VAV1's involvement in various immune system pathologies and cancers, underscoring its therapeutic potential. Understanding the structure-activity relationship of VAV1 concerning its GEF-dependent and GEF-independent functions provides a foundation for developing specific VAV1 GEF inhibitors. In this thesis, we employed different drug design strategies to identify specific inhibitors of VAV1 GEF activity. We identified two pockets of interest within key regulatory domains of the protein that enabled to perform a virtual ligand screening of millions of compounds. From the hits obtained in this screening, we created a library of compounds designed based on two promising hits, introducing chemical modifications to improve initial VAV1 GEF inhibition. The activity of these compounds, asserted through VAV1—RAC1 nucleotide exchange assays, showed significant improvement following various chemical modifications. Further characterization of lead compounds was performed using biochemical and cellular assays to assess their specificity for VAV1 GEF inhibition. Additional strategies in this project included the design and synthesis of a stapled peptide targeting VAV1, as well as high-throughput screening of two large compound libraries. Overall, this thesis presents a comprehensive exploration of strategies for identifying and validating VAV1 GEF inhibitors, revealing promising candidates for further investigation.

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LIST OF ABBREVIATIONS

Ac Acidic region

AITL Angioimmunoblastic T-cell lymphoma

ALCL Anaplastic large-cell lymphoma

BSA Bovine Serum Albumin

C1 C1 subtype zinc finger

CBLB CBL proto-oncogene B

CPS Counts per second

CSH3 C-terminal SH3

CTCL Cutaneous T-cell lymphoma

DH DBL homology

DMEM Dulbecco's modified Eagle's medium

DMSO Dimethyl sulfoxide

DTT Dithiothreitol

EDTA Ethylenediaminetetraacetic acid

EGTA Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

GAP Guanosine Activating Protein

GDI Guanosine Dissociation Inhibitor

GDP Guanosine diphosphate

GEF Guanosine nucleotide exchange factor

GST Glutathione S-transferase

GTP Guanosine triphosphate

HNSC Head and neck squamous cell carcinoma

HTS High throughput screening

JAK Janus kinase

JNK c-Jun N-terminal kinase

KO Knockout

LUAD Lung adenocarcinoma

MBP Maltose binding protein

MYOF Myoferlin

NFAT Nuclear Factor of Activated T cells

NMR Nuclear magnetic resonance

NOTCH1 Neurogenic locus notch homolog protein 1

NSCLC Non-small cell lung cancer

NSH3 N-terminal SH3

PBS Phosphate-buffered saline

PDB Protein data bank

PH Pleckstrin homology

PLCγ1 Phospholipase C gamma 1

PROTAC Proteolysis targeting chimera

PTCL Peripheral T cell lymphoma

RAC1 Ras-related C3 botulinium toxin substrate 1

RHOA Ras Homolog family member A

SAR Structure-activity relationship

SEM Standard error of the mean

SH Src homology

SOS Son of sevenless

SRF Serum response factor

TBS-T Tris-buffered saline and Tween 20

TCR T cell receptor

VLS Virtual ligand screening

WT Wild type

XRC X-ray crystallography

INTRODUCTION

1. THE VAV FAMILY OF RHO GUANINE NUCLEOTIDE EXCHANGE FACTORS

The VAV family is a group of phosphorylation-regulated proteins that work primarily as guanosine nucleotide exchange factors (GEFs) for RHO GTPases, while also playing complementary, GEF-independent roles in important signal transduction scenarios. This family is composed by three members in vertebrates (VAV1, VAV2 and VAV3), and one member in invertebrates, generally referred to as VAV [1]. The first member of this family was identified as an oncogene in 1989 [2] and over the past three decades, extensive research has elucidated the structures and functions of VAV family members, as well as their implications in disease [3].

1.1. VAV proteins as RHO GEFs

RHO GTPases cycle between an inactive, primarily cytosolic, guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state, typically associated with membranes, where their effector targets are located [4]. GTP binding activates RHO GTPases by inducing conformational changes that expose their effector domain switch regions, which increases their affinity for effector proteins. GEFs promote the activation of these proteins by catalyzing the release of GDP, which facilitates the incorporation of GTP into the guanosine nucleotide-binding pocket of GTPases. Conversely, GTPase-activating proteins (GAPs) inactivate RHO GTPases by accelerating their intrinsically low GTPase activity, thus favoring the transition to the inactive GDP-bound state. Additionally, guanosine nucleotide dissociation inhibitors (GDIs) regulate RHO GTPases by sequestering inactive GTPases in the cytosol, thus preventing their activation by GEFs [5] (**Figure 1**).

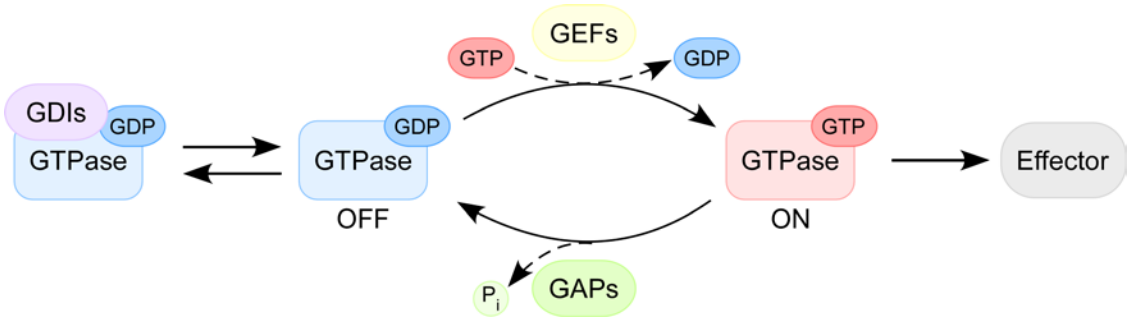

Figure 1. The RHO GTPase activation cycle. This illustration depicts the mechanisms of activation and inactivation of RHO GTPases through their regulators. Pi represents inorganic phosphate.

GEFs are essential for GTPase activation due to the high cytosolic Mg^{2+} concentrations that prevent spontaneous guanine nucleotide exchange in the cell. GEFs facilitate this exchange by displacing both the bound nucleotide and Mg^{2+} from RHO GTPases [6]. RHO GTPases regulate a wide range of cellular functions that vary with the cellular context, including general cell homeostasis roles (such as cell cycle progression and vesicle trafficking), cell morphology and dynamics (including migration and cell adhesion), metabolic processes (like reactive oxygen species production and glucose homeostasis), and morphogenesis (such as gene expression and neuronal development) [7–9].

GEF-mediated activation of RHO GTPases is a finely tuned spatiotemporal mechanism in cells. The tissue- and cell-specificity of GEF activity arises from the diversity this family has acquired through evolution, with over 80 distinct RHO GEFs identified in mammals [3]. Among these, VAV proteins display unique structural and regulatory features that set them apart. These characteristics include: (i) a catalytic DH-PH-C1 cassette requiring an allosteric conformational change for activation, (ii) a distinct activation mechanism involving tyrosine phosphorylation and autoinhibition, and (iii) an SH2 domain that enables the interaction with both transmembrane and cytoplasmic protein tyrosine kinases [1,3,10].

1.2. The VAV family protein structure

VAV family members are highly conserved proteins, comprising eight domains commonly found in signaling molecules: an N-terminal calponin homology domain (CH), an acidic region (Ac), a DBL homology domain (DH), a pleckstrin homology domain (PH), a C1 subtype zinc finger domain (C1 or ZF), an Src homology 3 domain (SH3), an Src homology 2 domain (SH2) and a second SH3 domain (**Figure 2A**). Notably, VAV proteins are the only known to integrate on the same molecule a DH-PH-C1 cassette and an SH2 region [10]. VAV1, VAV2 and VAV3 exhibit a high degree of structural similarity (**Figure 2A**), despite having only 50-60% sequence homology due to variations from redundant amino acid substitutions (**Figure 2B**).

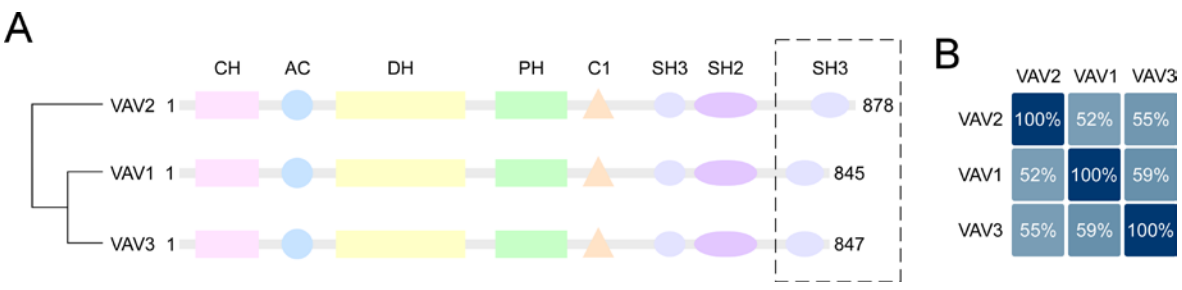

Figure 2. Multidomain protein structure of the VAV family. (A) The protein structures of the three mammalian VAV family members are depicted, along with their phylogenetic relationship shown on the left. The dashed box highlights the most significant structural difference observed among the family members. Domain abbreviations are provided in the main text. **(B)** A percent identity matrix derived from protein alignment of the three human VAV family members, obtained using UniProt.org.

The folding and structure of VAV proteins have been well studied and characterized. The DH-PH-C1 domains fold into a catalytic core, forming phosphorylation-dependent interactions with the CH-Ac domain and the C-terminal SH3 (CSH3) region, which are critical for VAV regulation [11] (**Figure 3**). The DH-PH-C1 core catalyzes the guanosine exchange reaction of RHO GTPases through direct interactions between the DH domain and the GTPases. Decades of mutagenesis, biochemical and structural studies have enabled detailed characterization of this domain. The DH domain contains three helices that specifically recognize and interact with residues in the two switch regions of RHO GTPases. Upon binding, the DH domain induces a conformational shift in the nucleotide-binding pocket of the GTPase, impacting the switch II region. This interaction triggers two critical events for GTPase activation: disruption of the Mg^{2+} binding site and destabilization of key residue-GDP interactions [6,12]. While the DH and C1 domains are essential for catalytic activity across all VAV members, the PH domain is not necessary for GEF activity in VAV2 and VAV3 [13,14].

Beyond the catalytic functions, other protein domains contribute to the overall activity of VAV proteins. The CH domain is involved in their non-catalytic functions, while the SH2 and CSH3 domains play critical roles in regulating VAV activity and localization. Notably, the N-terminal SH3 domain (NSH3) is not required for the activation of VAV family proteins, as demonstrated by mutagenesis experiments [15,16]. This finding aligns with the absence of NSH3 in invertebrate VAV proteins, suggesting that this domain likely arose as a gain-of-function through splicing events [1].

1.3. The regulatory mechanisms of VAV proteins

The tyrosine-phosphorylation state is currently the best characterized regulatory mechanism of VAV proteins. VAV autoinhibition is tightly controlled and dictates both catalytic and non-catalytic protein activity. In non-stimulated cells, VAV is found in an inactive closed configuration maintained by inhibitory interactions in the N-terminal region of the protein, where the CH and Ac domains are anchored to the previously described catalytic DH-PH-C1 cassette (**Figure 3B**). The blockage of the DH active site by the Ac helix provides an approximately tenfold suppression in physiological conditions.

Additionally, the binding of the CH domain to the N-terminal portion of the Ac finger and the DH-PH-C1 cassette thermodynamically restricts the inhibitory helix to the core, contributing an additional approximately tenfold inhibition. Together, these two events result in a cumulative catalytic suppression of around one hundredfold for VAV proteins in their autoinhibited physiological state [11].

The closed conformational state of VAV proteins transitions to an open state when key residues in the Ac domain—primarily Tyr174, and to a lesser extent Tyr142 and Tyr160—are phosphorylated. This phosphorylation releases the helix release from the DH core, allowing VAV to adopt an open conformation that facilitates substrate interaction. Phosphorylation of additional tyrosine residues in the C1 domain (Tyr541 and Tyr544) and the CSH3 domain (Tyr826, Tyr836 and Tyr841) also promotes this open conformation [3]. VAV activation is typically mediated by upstream tyrosine kinases, positioning VAV downstream of transmembrane receptors that contain either intrinsic or associated tyrosine kinase activity. For instance, in T cells, the most common phosphorylation events of VAV1 occur upon receptor stimulation by SRC- and SYK- family kinases. In this context, VAV1 activation is mediated by its SH2 and N-terminal SH3 (NSH3) domains. The SH2 domain facilitates the interaction of VAV1 with upstream protein tyrosine kinases, while the NSH3 domain plays a role in coupling with adaptor molecules [1,3,17].

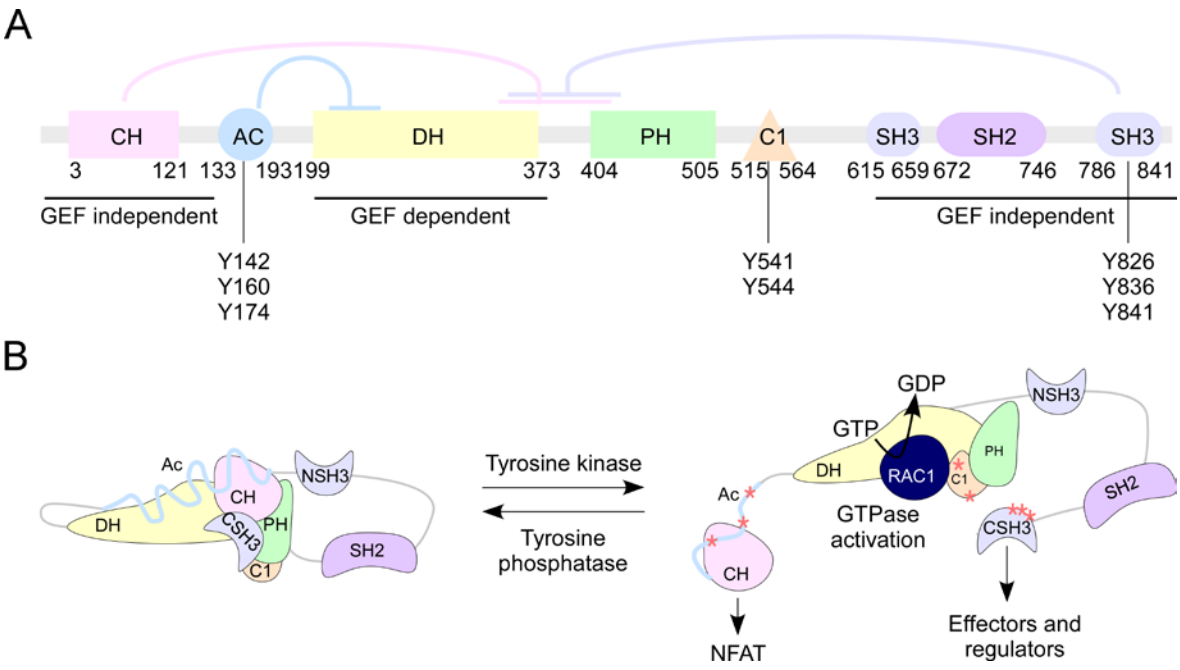

Figure 3. Structure and mechanism of autoinhibition in VAV protein family. (A) Depiction of the VAV1 structure, indicating the residues important for protein autoinhibition. **(B)** 3D schematic representation of VAV1 domains transitioning from a closed inactive conformation (left) to an open active conformation (right). Phosphorylated residues are denoted by dark orange asterisks. GEF-dependent activity is depicted as the exchange of GDP for GTP into substrate RAC1. GEF-independent functions include T cell activated nuclear factor (NFAT) activation through the CH domain and interactions with effectors and regulators via the CSH3 domain. Figure adapted from Bustelo, 2014 [1].

1.4. Physiological roles of the VAV family

The primary function of VAV proteins is to catalyze the nucleotide exchange of RHO GTPases, facilitating their transition from inactive (GDP-bound) to active (GTP-bound) conformation. The main substrates of VAV proteins include RAC1 and, to a lesser extent, RHOA [18–20]. By activating RAC1, VAV proteins trigger downstream effector signaling involving effector kinases such as PAK and JNK, and stimulate F-actin-regulated transcriptional cascades, including AP1 and serum response factor (SRF) [1] (**Figure 4**).

Despite their high structural homology, the three VAV members are not functionally redundant. The differential expression of VAV proteins accounts for tissue-specific functions in humans [21]. VAV1 is predominantly expressed in hematopoietic cells, where it activates RAC1 in response to signal transduction upon T cell activation. Its functions are primarily centered around the immune response, encompassing processes such as antigen recognition in T cells, phagocytosis in macrophages and superoxide production in neutrophils [16,17]. In contrast, VAV2 and VAV3 are more broadly expressed across tissues and also play important roles in development and cell function [1,3]. Research has highlighted the roles of VAV2 and VAV3 in the cardiovascular and nervous systems, respectively, where they play important roles in angiogenesis and development [22–25].

From a structural perspective, the functions of VAV proteins can be classified into GEF-dependent and GEF-independent functions, facilitated through different protein domains. The non-catalytic adaptor functions of VAV1 are primarily mediated by the CH and SH domains. The CH domain of VAV1 promotes the interaction of phospholipase Cγ1 (PLCγ1), an event that allows the subsequent downstream activation of the nuclear factor of activated T cells (NFAT) in lymphocytes. This pathway is essential for T cell activation, cytokine production and follicular helper T cell differentiation [26–29]. The CSH3 domain of VAV1 serves scaffolding functions with various proteins, including the CBL proto-oncogene B (CBL-B) E3 ubiquitin ligase. This interaction is important in buffering the active intracellular domain of NOTCH1 (ICN1) levels in T cells. (**Figure 4**). Additionally, VAV2 has been characterized within the immune system, where it performs similar roles to VAV1 but in different hematopoietic lineages. For instance, VAV2 can only stimulate NFAT in B lymphocytes but not in T cells, where VAV1 and VAV3 are more important [1].

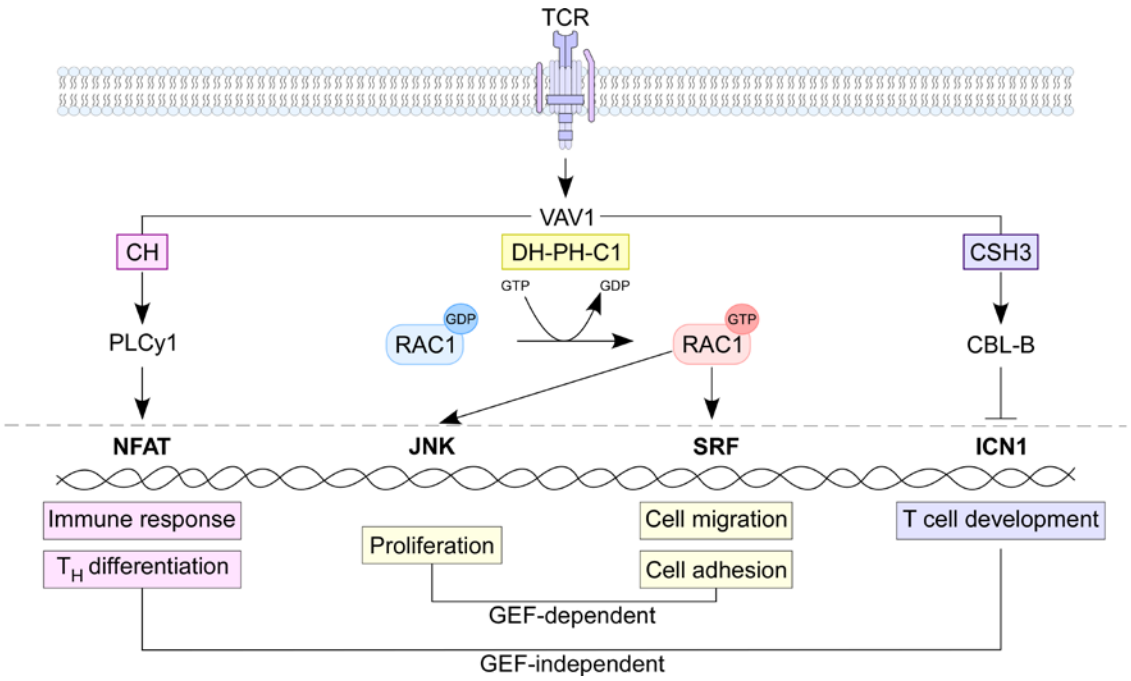

Figure 4. GEF-dependent and GEF-independent functions of VAV1 in T cells. This schematic illustrates the physiological roles of VAV1 in T cells. GEF-dependent functions encompass cell proliferation, migration and adhesion, while GEF-independent functions include immune responses, helper T cell differentiation and T cell development. The catalytic core of VAV1 activates RAC1, which in turn triggers downstream signaling pathways involving JNK and SRF. The CH domain of VAV1 activates the NFAT pathway through PLCφ1. The CSH3 domain of VAV1 interacts with CBL-B, which inhibits ICN1 signaling. The dashed grey line denotes the boundary between the cytosol and the nucleus.

1.5. VAV proteins in pathology

Given its hematopoietic expression and immune functions, it is not surprising to find VAV1 implicated in various immune disorders. Single nucleotide polymorphisms in VAV1 have been linked to the severity of diseases such as multiple sclerosis, rheumatoid arthritis or atherosclerosis (**Table 1**). Additionally, VAV1 has been described in immune responses like allograft tissue rejection and graft-versus-host disease [30].

Beyond its role in immunological dysfunctions, VAV1 has garnered interest in the cancer field since its discovery in 1989 due to its oncogenic activity observed in focus formation assays [2]. RHO GTPases play a major role in many aspects of tumorigenesis, often driven by GEF overexpression or the loss of GAP-mediated inactivation [31,32]. More recently, the role of VAV family proteins in cancer has been studied not only in terms of expression but also through oncogenic mutations (**Table 1**). Point mutations or translocations in VAV1 that disrupt key autoinhibitory interactions are frequently found in various tumor types, granting VAV1 a phosphorylation-independent activity that contributes to its oncogenic potential. Cancer genome studies in recent years have shown that VAV1

is frequently mutated in a number of non-Hodgkin lymphomas, particularly peripheral T cell lymphomas (PTCLs), and to a lesser extent in non-small cell lung cancer (NSCLC) (**Figure 5**) [33].

While VAV2 and VAV3 have been studied less extensively than VAV1, they are also associated with human pathologies, especially in cancer, where alterations in these proteins have been documented (**Table 1**).

Table 1. VAV family implications in human pathologies. This table summarizes the current clinical data on VAV proteins and their association with various immune disorders (light green) and cancers (light blue). Abbreviations include: PTCL; peripheral T cell lymphoma; ALCL, anaplastic large cell lymphoma; CTCL, cutaneous T cell lymphoma; LUAD, lung adenocarcinoma; NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell carcinoma.

VAV family member	Disease		Implications	References
VAV1	Multiple sclerosis		Overexpression and polymorphisms	[34,35]
	Rheumatoid arthritis			[36,37]
	Atherosclerosis			[38]
	Systemic lupus erythematosus			[39-41]
	Inflammatory bowel disease			[42,43]
	Periodonditis			[44]
	Autoimmune hepatitis			[45]
	Non-Hodgkin lymphomas	PTCL	Cancer driving mutations found	[33,46-52]
		ALCL		
		CTCL		
	Lung cancer	LUAD		[53]
		NSCLC		[53,54]
	Pancreatic cancer		Overexpression, worse survival correlation	[55]
	Neuroblastoma			[56]
VAV2	Rheumatoid arthritis		Signature established in patients	[57]
	HNSCC		Overexpression, poor prognosis correlation	[58]
	Breast cancer			[59,60]
	Adrenocortical carcinoma		Drives tumor cell invasion	[61]
VAV3	Breast cancer		Overexpression	[59]
	Colorectal cancer			[62]
	Prostate cancer			[63]
	Pancreatic cancer		Expression drives tumor aggressiveness	[64]
	Glioblastoma			[65]
	Ovarian cancer			[66]

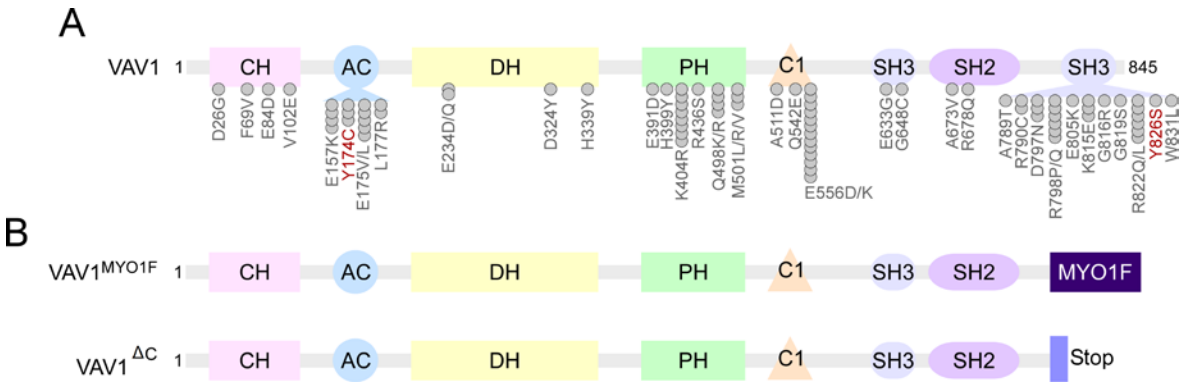

Figure 5. The mutational landscape of VAV1 in human cancers. This figure illustrates the VAV1 protein structure, highlighting the most prevalent mutations found in human cancers. **(A)** VAV1 missense point mutations in human cancers, with each dot representing one patient. Mutations affecting tyrosine residues important for VAV1 autoinhibition are marked in red. **(B)** Examples of common VAV1 oncogenic alterations, including a translocation (top) and a truncation (bottom). MYO1F refers to Myosin IF. Figure adapted from Robles et al., 2021 [67].

Experimental studies using murine models of VAV family proteins have proven invaluable in elucidating the GEF-dependent and GEF-independent pathways associated with tumorigenesis (**Table 2**). For instance, research has demonstrated that the disruption of the VAV1-regulated tumor suppressor pathway involving NOTCH1 is critical for the fitness of T cell acute lymphoblastic leukemia, particularly in the TLX^{+} clinical subtype [68]. This finding has revealed an unexpected tumor suppressor role of VAV1, prompting extensive investigations into its structure-function relationship and molecular mechanisms underpinning its dual roles in cancer. Such characterizations have been pivotal in advancing our understanding of VAV1's contributions to oncogenesis and its complex involvement in the pathogenesis of various malignancies [69,70].

Table 2. Overview of experimental data from VAV mouse models. This table summarizes information on VAV mouse models published to date. Immune-related findings are shaded in green, tumorigenic implications are marked in light blue, and cardiovascular effects are indicated in light red. Abbreviations include: T-ALL, T-cell acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia. Other abbreviations are defined in the main text.

Model type	VAV gene(s)	Phenotype	Mechanism	Reference
Knockout (KO) mice	$Vav1^{-/-}$	Impaired B1-cell development	Antigen-mediated proliferative defects, deficient TCR-dependent response	[71-73]
		Perypheral T cell lymphopenia		
		NK cell impairment	Inability to produce IL-4	[74,75]
		Resistance to experimental autoimmune encephalomyelitis	Impaired antigen priming	[76]
		Arthritis protection	T cell attenuation	[77]
		T-ALL development	ICN1 signaling hyperactivation	[68]
	$Vav2^{-/-}$	Cardiovascular defects	Chronic renin/angiotensin II and sympathetic nervous system stimulation	[22,24, 78-80]
	$Vav3^{-/-}$	Protection from p190-BCR-ABL induced B-ALL	Lower RAC2 activation	[81]
	$Vav2^{-/-}$ $Vav3^{-/-}$ double KO	Skin cancer protection	Keratinocyte autocrine/paracrine proliferation programs	[82]
	Vav triple KO	Impaired B and T cell function	TCR- and BCR-induced Ca^{2+} signaling impairment	[83]
		Arthritis protection	VAV/PLCy2 signaling downregulation	[84]
Gene edited mice	$VAV1^{R63W}$	Protection from experimental autoimmune encephalomyelitis	Decreased VAV1 phosphorylation in CD4+ T cells	[85]
	VAV1-MYOF fusion	PTCL development	Aggressive T cell lymphoproliferation	[86,87]
	$VAV1^{\Delta C}$		$VAV1^{\Delta C}$ in combination with Trp53 loss induces AITL signature upregulation	[88]
		Lung cancer progression acceleration	Encanced KRAS induced NSCLC	
	$VAV2^{\Delta C}$	Skin cancer development	Hyperplesia driven by c-Myc and YAP/TAZ	[58]
Orthotopic model	CD4+ $VAV1^{\Delta C}$	PTCL development	Triggered proliferative program in transformed cells	[33]
	$VAV2^{-/-}$ breast cancer	Lung metastasis protection	Reduced lung-specific metastasis signaling	[59]
	$VAV3^{-/-}$ breast cancer			

2. THE PROCESS OF DRUG DISCOVERY

2.1. Drug development

Drug design and development is an important research area that has evolved over centuries, beginning with with plant-based traditional medicine in ancient times and progressing into the field of medical chemistry by the 19th century. In the last century, the landscape has transformed dramatically due to the “omics revolution”, advances in computational chemistry, and the development of high throughput screening (HTS) techniques, which have pushed the boundaries of drug development further than ever before [89]. Despite these technological advances, drug development remains a lengthy and costly endeavor [90].

Drug development begins with drug discovery, where a vast array of compounds, numbering in the thousands to potentially hundreds of thousands, are screened with the aim of identifying promising candidates. This phase employs computational, biochemical and cellular biology techniques to generate lead compounds targeting specific biological pathways. The most promising leads then progress to the pre-clinical development stage, where their safety and toxicity are assessed *in vitro* and *in vivo*, typically using murine models. During this phase, critical information regarding compound safety, dosing and potential efficacy is collected [91]. Key pharmacokinetic and pharmacodynamic properties of the compounds are characterized by evaluating their absorption, distribution, metabolism and excretion (ADME) [92]. If these results are favorable, the compounds advance to clinical trials, which are rigorously regulated to address the specific needs of each drug, including patient selection, dosage and administration protocols. Clinical trials are conducted in phases, beginning with small-scale Phase I studies to assess safety and tolerability, followed by larger Phase II and III studies, designed to evaluate efficacy and further monitor safety, with increasing participant numbers and testing durations. The drug development process concludes with data review and approval from federal regulatory agencies, followed by commercialization and ongoing post-market safety monitoring [93].

2.2 Drug discovery strategies

Drug discovery, the initial phase of drug development discussed previously, is underpinned by basic research across various scientific disciplines. Modern drug research employs diverse strategies that integrate insights from biology, biochemistry, pharmacology, mathematics and computational modelling [89]. The process begins with the identification

of a target molecule, typically a protein, which serves as the focal point for drug design. Strategies for drug discovery can be classified into high-throughput screening (HTS) and structure-activity relationship (SAR) drug design (**Figure 6**). Both approaches aim to identify lead compounds—chemical structures that demonstrate activity and selectivity in biochemically relevant screening assays. These lead compounds require validation and can be further optimized and characterized.

In the HTS strategy, large chemical libraries are screened against the chosen target to identify promising candidates. Conversely, SAR focus on drug design tailored specifically to the target molecule, incorporating detailed structural and functional data. Often, these strategies are combined: hits from HTS can inform and refine the SAR process, while active compounds identified in library screenings can be enhanced using structure-activity models of the target. Both SAR results and HTS leads undergo rounds of chemical synthesis and testing. This interactive cycle of synthesis and testing continues until a drug with the desired activity is successfully developed.

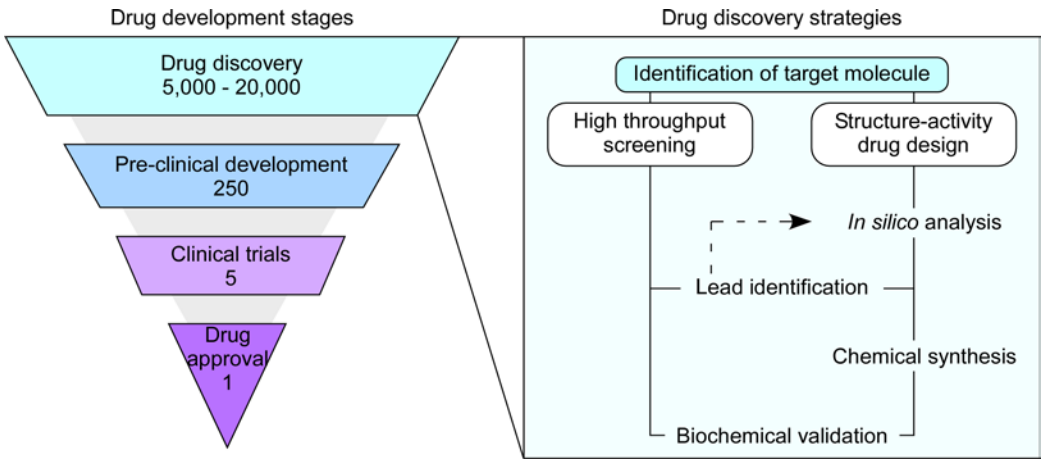

Figure 6. The process of drug discovery. The left panel illustrates the stages of the drug development, indicating the approximate number of molecules that are tested at each stage. The right panel outlines the main drug discovery strategies, highlighting their interconnections and interactions. Figure adapted from *Technology Networks* [94].

2.2.1 High throughput screening of compounds

HTS is a widely used method for the initial screening of bioactive compounds. HTS can be categorized into two main types: biochemical assays and cell-based assays. In biochemical HTS, thousands to millions of small molecules are presented to a target of interest and evaluated using biochemical assays, typically conducted in 384-well plates. This format strikes a balance between screening volume, sample size, and equipment costs. Common

optical detection methods include absorbance, fluorescence, and luminescence. Fluorescent based assays, which measure signal changes during enzymatic reaction, are particularly prevalent in HTS. Examples of fluorescence-based HST methods include total fluorescence intensity (FLINT), fluorescence anisotropy/polarization (FA/FP), and fluorescent resonance energy transfer (FRET). In recent years, binding-based technologies for assessing drug-protein interactions have gained traction, with microarray technology being optimized to screen targets against extensive libraries of immobilized compounds on solid supports. [90,95]. However, these assays are not without limitations; challenges such as false positives, low-specificity in target binding and a lack of standardized HTS data reporting persist [96]. While the first two issues should be addressed in subsequent validation stages, there remains a pressing need for improved normalization and accessibility of HTS data.

2.2.2. Structure-activity drug design

Omics data have contributed significantly to the refinement of the drug discovery process. The cloning and expression of disease-specific target proteins have greatly enhanced the specificity of drug design, while patient transcriptomic data now guide the development of more tailored treatments, advancing towards “personalized medicine” [89]. Importantly, advances in the field of structural biology with methodologies like X-ray crystallography (XRC), nuclear magnetic resonance (NMR) and cryo-electron microscopy have vastly remodeled the field of medicinal chemistry. These methods provide detailed structural insights into protein targets, enabling researchers to convert initial screening hits into promising leads or drug candidates. Additionally, structural biology techniques now reveal the 3D configurations of protein-ligand complexes, offering a framework for optimizing ligands and screening small-molecule libraries. In recent years, fragment-based drug discovery using XRC and NMR has gained popularity as a powerful tool that not only identifies ligands, but also provides experimental data on the binding modes of molecular fragments, guiding more precise drug design [97].

The availability of high-resolution protein 3D structures has greatly propelled computational approaches in drug discovery, particularly molecular docking. This structure-based *in silico* method predicts the optimal orientation of a ligand within a predefined protein receptor or binding pocket by applying energy-based scoring models. Each potential ligand orientation is evaluated based on the strength of non-covalent interactions, providing a theoretical binding affinity that helps identify promising candidates [98]. Widely used

docking programs are AutoDock, Vina, DOCK and GOLD vary in algorithms and scoring functions, each offering unique strengths.

Molecular docking has paved the way to virtual ligand screening (VLS) techniques, which are often the first *in silico* step in drug discovery. VLS complements and accelerates traditional HTS techniques by reducing the number of false positives and negatives that can arise due to issues like compound solubility and stability [99]. Today, various commercial virtual libraries are available, ranging in size, origin and structural diversity, allowing for tailored selection based on screening objectives. For instance, drug repurposing libraries can expedite the lengthy and costly stages of drug development discussed in **Section 2.1**. Properly defining the target is essential for effective virtual screening, as most processes rely on high-throughput molecular docking to assess potential binding interactions.

2.3. Future prospects in drug design

Recent advances in computational chemistry hold promise for alleviating the low efficacy and high costs associated with drug design and discovery. For instance, molecular docking methods have significantly advanced over the past decade, paving the way for molecular dynamics-based approaches. Unlike traditional docking, molecular dynamics stimulations integrate the thermodynamics and kinetics of ligand binding and unbinding, providing a more realistic and physiologically relevant view of protein behavior [100]. The rapid generation of omics data underscores the need for effective integration methods, and artificial intelligence (AI) and machine learning technologies hold substantial promise in meeting this challenge [101]. Recent breakthroughs in AI, such as AlphaFold—a tool for predicting 3D protein structures —have demonstrated significant potential in advancing the field of structural biology, providing insights that could accelerate drug discovery and personalized medicine.

Despite these computational advances, traditional small-molecule inhibition still faces notable challenges and limitations. Proteins with broad active sites, inaccessible binding pockets, or smooth surfaces are often deemed “undruggable” due to their structural complexity, which hinders chemical drug design. Computational approaches may also struggle with these targets, as they rely on well-defined binding pockets for accurate predictions [98,100]. To address these issues, new biochemical strategies have emerged over the past decade, diverging from standard small-molecule compounds. Peptide-based inhibitors for targeting protein-protein interactions (PPIs) and targeted protein degradation (TPD) technologies exemplify innovative approaches gaining traction in protein inhibition.

Peptide-based inhibitors have emerged as a powerful tool for targeting PPIs. Kown as “stapled peptides” due to their structural modifications, these peptides incorporate chemical constrains, or “staples”, that preserve their alpha-helical conformation, protecting them from protease degradation and enhancing their overall biophysical properties, including cellular stability and uptake [102,103]. Although stapled peptide design requires structural knowledge of the target protein, it offers a solution for cases where standard small molecules are ineffective due to inaccessible binding pockets. Stapled peptides can also selectively disrupt specific interactions within multidomain proteins, allowing for targeted inhibition of particular effector PPIs. Recent examples of stapled peptides advancing to clinical trials include murepavadin for pneumonia treatment, balixafortide for metastatic breast cancer, and pegcetacoplan, an approved treatment for paroxysmal nocturnal hemoglobinuria [104,105].

Another promising biochemical approach addressing the challenge of protein druggability that has gained significant attention in the past decade is TPD. Proteolysis-targeting chimeras (PROTACs) are a class of protein degraders that consist of two ligands connected by a linker: one ligand binds to the target protein, while the other recruits an E3 ubiquitin ligase. The simultaneous binding of these ligands facilitates the ubiquitylation of the target, leading to is subsequent proteasomal degradation [106]. Although PROTACs require a known binding site on the target protein, they offer a viable solution for proteins with inaccessible cavities. Furthermore, PROTACs are recycled after each proteolytic event, allowing for significantly lower dosages and reduced frequency of administration. This technology also holds promise for overcoming drug resistance, a common hurdle encountered in cancer drug development [107].

3. VAV1 AS A TARGET FOR DRUG DISCOVERY

3.1. Challenges in the GTPase drug discovery field

Small GTPases have long been considered “undruggable” in cancer research due to their limited stable cavities, primarily restricted to their nucleotide-binding pocket. Their sub-nanomolar binding affinity for GTP or GDP, coupled with the physiological micromolar concentration of these nucleotides, complicates the targeting of these proteins with small molecule modulations, in contrast to protein kinases. Consequently, most current cancer therapies aimed at GTPases focus on inhibiting their downstream effectors [108]. Among the GTPases, the RAS superfamily has attracted significant attention due to its critical role in cancer, with over 50 inhibitors approved for RAS-mutant tumors. However, targeting RAS mutations has proven to be a costly endeavor, often challenged by issues of drug resistance and off-target side effects [109,110]. In recent years, strategies to suppress RAS-mutant tumor growth frequently involved targeting RAS effectors, with RAF, MEK or mTOR inhibitors being the most common examples [111]. Notably, the identification of a cryptic allosteric pocket in the switch II domain of K-RAS mutants [112–115] established the foundation for the clinical development of covalent and selective $KRAS^{G12C}$ inhibitors, which have since received approval for the treatment non-small cell lung cancer [116,117].

For the RHO family of cancer mutations, directly targeting RHO GTPases and their effectors remains the predominant strategy in drug development as well. Most compounds targeting RHO activation focus on blocking GEF binding, such as CDC42 inhibitors. Although several inhibitors have been developed for RHO, CDC42, and their respective ROCK and PAK effectors, inhibitors specific to RAC1 remain limited. This landscape may evolve in upcoming years, especially in light of the recent paradigm shift in K-RAS drug development, a breakthrough that has demonstrated potential for application in RHO GTPases [118]. Additionally, there has been a notable increase in efforts to target RHO GTPase regulators rather than their downstream effectors, creating new avenues for drug development that diverge from traditional targeting targets [108].

3.2. Current inhibitors against RHO GEFs

Despite their significant implications in cancer development, inhibitors targeting RHO GEFs remain notably underrepresented in the drug discovery landscape [31]. HTS technologies have facilitated the identification of some RHO GEF inhibitors, including RNA aptamers for TIAM1 [119], aptamer-derived peptides for TRIO [120], and small molecule inhibitors for ARHGEF12 [121,122] (**Figure 7**). However, the development of RHO GEF inhibitors through SAR drug design approaches has been limited, likely due to difficulties in identifying

viable target protein pockets. For instance, the small molecule IODVA1 was initially identified in a virtual library screening targeting oncogenic RAS^{G60A} [123], and later characterized as a VAV3 inhibitor [124], evidencing the importance of establishing accurate binding pockets during the *in silico* identification of lead compounds. Furthermore, drug-repurposing efforts have yielded interesting findings, such as the identification of the immunosuppressive agent Azathioprine as a VAV1 inhibitor [125]. However, its off-target effects could difficult its potential therapeutic application in cancer treatment. Overall, the pursuit of effective RHO GEF inhibitors remains an area ripe for exploration, with the potential to unlock new therapeutic strategies in oncology.

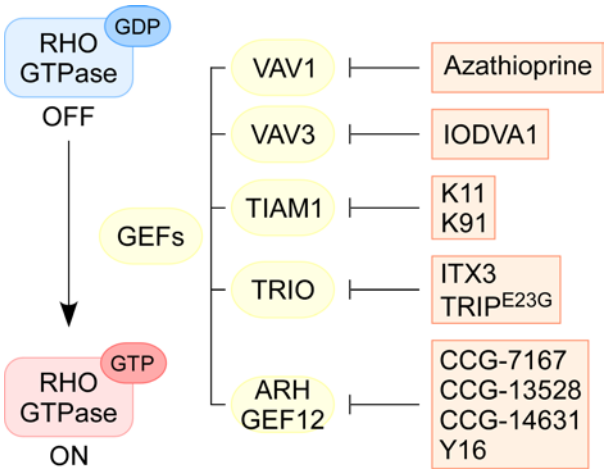

Figure 7. Current inhibitors against RHO GEFs. Summary of the current RHO GEF inhibitors, represented in orange boxes, and their RHO GEF targets, represented in yellow.

3.3. The therapeutic value of VAV1

The pathological implications of VAV1 alterations revealed over the past decades underscore its potential as a therapeutic target in both immune disorders [126] and cancer [127]. Research using extensive murine models has clarified the mechanisms by which VAV1 contributes to human malignancies, laying the groundwork for targeted drug design strategies. Two significant challenges that previously hindered SAR drug design for VAV1 have now been overcome.

First, advances in structural protein characterization have enhanced the modeling of GTPase-GEF complexes, facilitating more precise drug design and increasing the potential for specificity in targeting VAV1. Second, recent insights into the dual roles of VAV proteins in cancer have uncovered new possibilities for therapeutic intervention [68–70]. The detailed characterization of both GEF-dependent and GEF-independent regulatory mechanisms associated with VAV1 has expanded the therapeutic landscape, highlighting

the benefits of selectively targeting its catalytic activity while preserving its CSH3-mediated tumor suppressor functions.

Moreover, the evolving understanding of VAV1's mutational landscape further reinforces its status as an attractive candidate for novel targeted therapies. As research continues to delineate the complex roles of VAV1 in tumor biology, the potential for developing innovative therapeutic strategies targeting this protein becomes increasingly promising. Exploring these avenues could lead to new treatment options for patients with malignancies associated to VAV1 dysregulation, an area that has yet to be thoroughly explored in biomedical research.

OBJECTIVES

The main objectives of this Ph.D. thesis are:

1. To develop and evaluate complementary strategies for inhibiting VAV1 based on: *in silico* drug screening, peptide-mimetics, and conventional compound library screening.

2. To isolate and subsequently optimize inhibitory leads to block the catalytic activity or the allosteric activation step of VAV1.

3. To validate the most promising VAV1 inhibitors *in vitro.*

METHODS

In silico analyses

Protein structures were obtained from the PDB and visualized using BIOVIA Discovery Studio 2024. Molecular docking was performed in collaboration with Dr. Xavier Rovira of the Medicinal Chemistry and Synthesis group, led by Dr. Amadeu Llebaira at the Advanced Chemical Institute of Catalunya (IQAC) in Barcelona, Spain, also performed using BIOVIA Discovery Studio 2024. Virtual ligand screening of the ZINC database was performed by Dr. Lorenzano-Menna at Quilmes University in Buenos Aires, Argentina.

Compounds

Compound hits from the virtual ligand screening were sourced from the ENAMINE store, with reference codes noted in figures. Compounds from MCS libraries were designed and synthesized by Dr. Carme Serra of the Medicinal Chemistry and Synthesis group at IQAC. Tested compounds from large library screenings include: quinacrine dihydrochloride (Cat. #Q3251; Merck), daunorubicin hydrochloride (Cat. #30450; Merck) and pyrvinium pamoate (Cat. #P0027; Merck). All compounds were diluted in DMSO to 20 mM stock solutions, stored at -20ºC, and shielded from light.

Purification of MBP fusion proteins

Exponentially growing cultures of *E. coli* DH5α cells transformed with MBP-VAV1 plasmid were induced with 0.3 mM IPTG (Cat. # I5502; Sigma) at 25°C for 90 min. Cells were centrifuged at 6,000 rpm for 10 min at 4ºC and resuspended in lysis buffer [10 mM sodium phosphate, 0.25% (w/w) Tween-20, 30 mM NaCl, 10 mM β–mercaptoethanol, 10 mM EDTA, 10 mM EGTA]. The samples were frozen overnight, thawed in ice, disrupted by sonication on ice, and centrifuged again. The supernatant containing MBP-VAV1 was mixed and incubated with amylose beads (Cat. #E8021; New England Biolabs) at 4º C in rotation for 1-2 hours. Beads were stacked into 10 ml columns and MBP-VAV1 was eluted with 10 mM maltose in column buffer (20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4). 1 ml fractions were serially collected and protein concentration was measured using a NanoDrop Spectrophotometer (Thermo Fished Scientific). The fractions containing more than 0.1 mg/ml of protein were unified and dialyzed in dyalisis buffer (100 mM NaCl, 10% Glycerol, 1 mM EDTA, 20 mM Tris-HCl, 1 mM DTT) overnight at 4ºC. The next day, samples were aliquoted, frozen in dry ice and conserved at – 80ºC until further use. Samples from each purification step were analyzed by PAGE.

Purification of GST fusion proteins

Exponentially growing cultures of *E. coli* DH5α cells transformed with GST-RAC1 plasmid were induced with 0.3 mM IPTG (Cat. # I5502; Sigma) at 30°C for 60 min, centrifuged at 6,000 rpm for 10 min at 4ºC and resuspended in lysis buffer [PBS, 1/2 Complete-EDTA free protease inhibitor tablet (Cat #11697498001; Sigma), 1 mM DTT, 1% (w/w) Titron X-100, 10 mM $MgCl_2$]. The samples were frozen overnight, thawed in ice, disrupted by sonication on ice, and centrifuged again. The supernatant containing GST-RAC1 was mixed and incubated with Sepharose beads (Cat. #4B200; Sigma) at 4º C in rotation. Beads were stacked into 10 ml columns and GST-RAC1 was eluted with 10 mM reduced gluthation in column buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1 mM EDTA). 1 ml fractions were serially collected and protein concentration was measured using a NanoDrop Spectrophotometer (Thermo Fished Scientific). The fractions containing more than 0.1 mg/ml of protein were unified and dialyzed in dyalisis buffer (100 mM NaCl, 10% Glycerol, 20 mM Tris-HCl, 1 mM DTT, 10 mM $MgCl_2$) overnight at 4ºC. The next day, samples were aliquoted, frozen in dry ice and conserved at – 80ºC until further use. Samples from each purification step were analyzed by PAGE.

Nucleotide exchange assays

Reactions were conducted in black 96-well plates at 50 µl volume in rection buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$). Prior to plate preparation, GST-RAC1 was incubated with BODIPY-FL-GDP (Cat #G22360; Thermofisher) in a 4:1 ratio at 4ºC for 1 hour. For the nucleotide release assay, samples were prepared by mixing BODIPY-FL-GDP loaded GST-RAC1 (GST-RAC1-BP) (0.4 µM), MBP-VAV1 (0.8 µM), and 1 µl of tested inhibitor in each well. Negative control was prepared by adding GST-RAC1-BP alone with 1 µl of DMSO, and positive control was prepared mixing GST-RAC1-BP, MBP-VAV1 and 1 µl of DMSO. Plates were taken to TECAN Infinite 200 microplate reader and 50 µM GDP mix was added using a multi-channel pipette right before starting data acquisition. Reactions were measured for 1 hour using 485 nm excitation and 535 nm emission waves in intervals of 12 seconds. For the nucleotide incorporation assay, the same procedure was followed using unloaded GST-RAC1 and starting the reaction with excess BODIPY-FL-GDP. Data was analyzed with Excel (Office 2016; Microsoft) and GraphPad Prism software (version 9.0; Dotmatics), using exponential decay and dose-response three parameter Log (inhibitor) vs. response non-linear fit equations to calculate K_{obs} and IC_{50} values.

High throughput screening of compounds

The Prestwick chemical library of FDA and EMA approved drugs (L1), and a representative subset of the Innopharma structurally diverse chemical library (L2) were screened by the

group of Dr. Mabel Loza García in the University of Santiago de Compostela (USC), in Santiago, Spain, by adapting the nucleotide release assay previously described to HTS technologies. First, test compounds and proteins provided were added to a 384-well plate using an Echo 550 dispenser. Then, a Hamamatsu FDSS7000 reader was used to record a baseline reading of 75 seconds, and then the GDP mix was added while recording fluorescence intensity in real time. An exponential decay equation was used to fit the raw data and calculate K_{obs} values, with hit threshold fixed at mean % inhibition +2S (L1) and at mean % inhibition +3SD (L2). IC_{50} values were calculated using a three parameter Log (inhibitor) vs. response non-linear fit equation for the dose-response experiments.

Cell culture

NIH-3T3 cells were obtained from ATCC and cultured in DMEM containing 10% fetal bovine serum, 1% L- glutamine, penicillin (10 µg/ml) and streptomycin (100 µg/mL) and maintained at 37 ºC and a 5% CO2 humidified atmosphere. All the reagents were obtained from Gibco.

Jurkat cells were obtained from the ATCC and grown in RPMI–1640 medium supplemented with 10% fetal calf serum, 1% L–glutamine, penicillin (10 µg/ml) and streptomycin (100 µg/ml). All cell lines were maintained at 37ºC and a 5% CO2 humidified atmosphere.

Plasmids expressing $Vav1^{WT}$ (pJLZ52) and $Vav1^{Y174C}$ (pRHL02) were previously described [15,17,33,49]. The plasmid encoding VAV1-CAAX (pSRF93) combines whole VAV1 with the most C–terminal sequence (CVLS) of the H–Ras CAAX box and was also previously described [15,128].

Cell proliferation assays

For cell proliferation assays, 5.000 cells (NIH-3T3) or 20.000 cells (Jurkat) were seeded in 96 well plates in 100 µl of medium. All compound treatments were prepared in a master mix in cell medium to add another 100 µl to each well, for a final volume of 200 µl. Untreated cells were administered the highest concentration of the vehicle (1 µl of DMSO per well). For Jurkat proliferation assays, plates were previously coated with 1:100 poly-L-lisine (Cat #5048, Advanced Biomatrix, 0.1 mg/ml). The Incucyte SX5 Live-Cell Imaging system was used to acquire and analyze images at initial time and final time (96 hours after compound treatment). Four images were acquired within each p96 well. Incucyte Live-Cell Analysis cell-by-cell confluence mask software was used for data quantification. All data was normalized to the initial time within each individual well. All experiments included technical duplicates and were performed in at least three independent assays.

Luciferase reporter assays

For either SRF, $3x10^6$ of exponentially growing Jurkat cells were co-electroporated (1700 V, 20 ms, 1 pulse) with pSRF-luc reporter vector (4 µg and 3 µg, respectively), the pRL-SV40 *Renilla* vector (1 µg), and 10 µg of the appropriate Vav1-encoding vector using the Neon™ Transfection System (Invitrogen). 4-5 hours after transfection, cells were treated with the correspondent amount of the indicated VAV1 inhibitor. Also, SRF inhibitors CCG_222740 (Cat #HY-121750, MedChemExpress, 20µM) and CCG_1423 (Cat #555558, Sigma-Aldrich, 20µM) were used as control of inhibition in the case of SRF activation. 48 upon transfection the cells were then lysed with Passive Lysis Buffer (Cat No. E1960, Promega) and luciferase activities determined using the Dual Luciferase Assay System (Cat No. E1960, Promega) in a GloMax Navigator System (Promega). We normalized the firefly luciferase activity obtained in each experimental point to the activity of the *Renilla* luciferase obtained in each sample.

Western blotting

To determine the abundance of proteins, cells were washed with PBS solution and lysed in lysis buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100 (Cat. number X100, Sigma), 1 mM Na_3VO_4 (Cat. number S6508, Sigma), 10 mM β-glycerophosphate (Cat. number 50020, Sigma-Aldrich), and a cocktail of protease inhibitors [Cømplete, Cat. No. 05056489001, Roche]). Cellular extracts were precleared by centrifugation at 14,000 rpm for 10 minutes at 4°C, denaturalized by boiling in SDS-PAGE buffer, separated electrophoretically, and transferred into nitrocellulose filters (Cat. No. 2022-04-26, Thermo Fisher) using the iBlot Dry Blotting System (Thermo Fisher). Membranes were blocked in 5% bovine serum albumin (Cat. No. A4503, Sigma-Aldrich) in TBS-T (25 mM Tris–HCl [pH 8.0], 150 mM NaCl, 0.1% Tween-20 (Cat. number P7949, Sigma)) for at least 1 hour and then incubated 2 hours at room temperature or overnight at 4°C with the proper primary antibodies. Then, membranes were washed with TBS-T, incubated with the appropriate fluorescent-labeled secondary antibody (1:5000 dilution, LI-COR) for 45 minutes at room temperature, and washed as above. Fluorescent bands were visualized using a Odyssey XF Imager (LI-COR). Primary antibodies used included those to the Vav1 DH (homemade, 1:10,000 dilution), and tubulin α (Cat. No. CP06-100UG, Calbiochem; 1:2,000 dilution).

Image processing

All images and figures were assembled and processed for final presentation with Canvas Draw X Draw 2017 for Windows software.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism software (version 9.0; Dotmatics, Boston, MA, USA). The number of replicates and the statistical test used in each case are indicated in the figure legends. Parametric distributions were analyzed using Student's *t*-test. In all cases, values were considered significant when $P \leq 0.05$. P values have been depicted using the * (when < 0.05), ** (when < 0.01), and *** (when < 0.001) notation. Data are represented as the mean ± SEM.

CONCLUSIONS

The main conclusions of this Ph.D. thesis were:

1. *In silico* and *in vitro* screening methods, alongside with site-directed peptide mimetics approaches, have been shown to be effective strategies for the identification of inhibitors targeting autoinhibitory and allosteric sites of VAV1.

2. A total of 15 compounds exhibiting significant VAV1 GEF inhibitory activity were identified through targeted chemical modifications of *in silico* hits, with nitro substitutions strongly favoring compound activity.

3. The characterized inhibitors have been shown to be specific for VAV1 and not for the RAS GEF SOS1.

4. The most promising VAV1 inhibitors have been validated through preliminary tests in VAV1-transformed NIH-3T3 cell lines and in VAV1-dependent T cell responses in Jurkat cells.

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APPENDIX: RESUMEN EN CASTELLANO

ABSTRACT

VAV1 es un GEF de RHO que se expresa en células hematopoyéticas y activa las GTPasas RHO, principalmente RAC1, desempeñando roles cruciales en la respuesta inmune, la señalización celular y el desarrollo de células T. Estudios recientes han elucidado la implicación de VAV1 en diversas patologías del sistema inmunológico y en cánceres, destacando su potencial terapéutico. Comprender la relación entre la estructura y la actividad de VAV1 en sus funciones dependientes e independientes de GEF proporciona una base para desarrollar inhibidores específicos de VAV1 GEF. En esta tesis, empleamos diferentes estrategias de diseño de fármacos para identificar inhibidores específicos de la actividad GEF de VAV1. Identificamos dos sitios de interés dentro de dominios regulatorios clave de la proteína, lo que permitió realizar un cribado virtual de millones de compuestos. A partir de los resultados obtenidos, creamos una biblioteca de compuestos diseñados basados en dos hallazgos prometedores, introduciendo modificaciones químicas para mejorar la inhibición inicial de VAV1 GEF. La actividad de estos compuestos, confirmada a través de ensayos de intercambio de nucleótidos VAV1—RAC1, mostró una mejora significativa tras diversas modificaciones químicas. Se realizó una caracterización adicional de los compuestos candidatos mediante ensayos bioquímicos y celulares para evaluar su especificidad en la inhibición de VAV1 GEF. Otras estrategias en este proyecto incluyeron el diseño y la síntesis de un péptido con estructura anclada que se dirige a VAV1, así como un cribado de alto rendimiento de dos grandes bibliotecas de compuestos. En general, esta tesis presenta una exploración completa de estrategias para identificar y validar inhibidores de VAV1 GEF, revelando candidatos prometedores para investigaciones futuras.

RESUMEN DE LA INTRODUCCIÓN

La familia VAV de factores de intercambio de nucleótidos de guanina RHO

La familia VAV es un grupo de proteínas de transducción de señales reguladas por fosforilación que funcionan principalmente como factores de intercambio de nucleótidos de guanina (GEF) para las RHO GTPasas, aunque también desempeñan una variedad de funciones independientes de GEF en importantes escenarios de transducción de señales. Esta familia está compuesta por tres miembros en vertebrados (VAV1, VAV2 y VAV3) y un miembro en invertebrados, generalmente conocido como VAV. La estructura y funciones

de los miembros de la familia VAV, así como sus implicaciones en enfermedades, han sido estudiadas y complementadas durante las últimas tres décadas.

Las RHO GTPasas alternan entre un estado inactivo, principalmente citosólico, unido a guanosina difosfato (GDP) y un estado activo unido a guanosina trifosfato (GTP), generalmente asociado con membranas, donde se encuentran los efectores. La unión de GTP activa las RHO GTPasas al cambiar la exposición del dominio efector (regiones de cambio), aumentando así la afinidad por las proteínas efectoras. Los GEF promueven la activación de estas proteínas al catalizar la liberación de GDP y facilitar la incorporación de GTP en el bolsillo de unión de nucleótidos de las GTPasas. Por el contrario, la inactivación de las RHO GTPasas es mediada por proteínas activadoras de GTPasa (GAP), que aceleran su actividad intrínseca de GTPasa, favoreciendo así la transición al estado inactivo unido a GDP. Los inhibidores de disociación de nucleótidos de guanina (GDI) son reguladores adicionales de las RHO GTPasas que secuestran GTPasas inactivas en el citosol, evitando su activación por los GEF.

Los GEF son necesarios para la activación de las GTPasas debido a las altas concentraciones de Mg2+ en el citosol, que impiden el intercambio espontáneo de nucleótidos de guanina en la célula. Los GEF facilitan el intercambio de nucleótidos desplazando tanto el nucleótido unido como el Mg2+ de las RHO GTPasas. Las RHO GTPasas desempeñan una variedad de funciones celulares que varían según el contexto celular, incluyendo funciones generales de homeostasis celular, morfología, funciones metabólicas y morfogénesis.

Los miembros de la familia VAV son proteínas altamente conservadas estructuralmente compuestas por ocho dominios: un dominio de homología calponina N-terminal (CH), una región ácida (Ac), un dominio de homología DBL (DH), un dominio de homología pleckstrina (PH), un dominio de dedo de zinc tipo C1 (C1 o ZF), un dominio de homología 3 de Src (SH3), un dominio de homología 2 de Src (SH2) y un segundo dominio SH3. Estos dominios son comunes en muchas moléculas de señalización. VAV1, VAV2 y VAV3 comparten una gran similitud estructural, aunque la alineación de secuencias proteicas muestra una homología de 50-60% de pares de bases explicada por diferencias en reemplazos redundantes de aminoácidos.

El plegamiento de las proteínas VAV ha sido bien estudiado y caracterizado. Los dominios DH-PH-C1 se pliegan juntos en un núcleo catalítico y establecen interacciones dependientes de fosforilación con el dominio CH-Ac y la región SH3 C-terminal (CSH3) que son esenciales para su regulación. El módulo DH-PH-C1 cataliza la reacción de

intercambio de guanosina de las RHO GTPasas mediante interacciones del dominio DH con las GTPasas. El dominio DH contiene tres hélices que reconocen e interactúan con residuos en las dos regiones de cambio de las RHO GTPasas. Cuando el dominio DH de VAV se une a la GTPasa, induce un cambio conformacional en el bolsillo de unión de nucleótidos que afecta a la región *swtich II*. Esta interacción lleva a dos eventos críticos para la activación de la GTPasa: la perturbación del sitio de unión del Mg2+ y la pérdida de la interacción clave entre el GDP y el residuo esencial. Mientras que los dominios DH y C1 son necesarios en todos los miembros de VAV para la actividad catalítica, el dominio PH no es esencial para la actividad GEF en VAV2 y VAV3.

Mecanismos regulatorios de las proteínas VAV

El estado de fosforilación de tirosina es actualmente el mecanismo de regulación mejor caracterizado de las proteínas VAV. La autoinhibición de VAV está estrechamente controlada y dicta la actividad catalítica y no catalítica de la proteína. En células no estimuladas, VAV se encuentra en una configuración cerrada e inactiva, mediada por interacciones inhibitorias que se establecen en la región N-terminal de la proteína, donde los dominios CH y Ac están anclados al núcleo catalítico DH-PH-C1. El bloqueo del sitio activo DH por la hélice Ac proporciona una supresión de la actividad proteica de aproximadamente 10 veces en condiciones fisiológicas. Además, la unión del dominio CH a la parte N-terminal del dedo Ac y al núcleo DH-PH-C1 restringe termodinámicamente la hélice inhibidora al centro catalítico, contribuyendo a una inhibición de aproximadamente 10 veces. Juntos, estos dos eventos proporcionan una supresión catalítica de aproximadamente 100 veces de las proteínas VAV en condiciones fisiológicas autoinhibidas.

El estado conformacional cerrado de las proteínas VAV se revierte a un estado abierto cuando se fosforilan residuos clave en el dominio Ac (principalmente Tyr174, y en menor medida Tyr142 y Tyr160). Esta fosforilación provoca la liberación de la hélice autoinhibutoria del dominio DH, permitiendo que VAV adopte una conformación abierta que facilita su interacción con el sustrato. La fosforilación de residuos adicionales de tirosina en los dominios C1 (Tyr541 y Tyr544) y CSH3 (Tyr826, Tyr836 y Tyr841) también favorece la conformación abierta. La activación de VAV suele estar mediada por quinasas de tirosina aguas arriba, ubicando a VAV aguas abajo de receptores transmembrana que contienen actividad de tirosina quinasa intrínseca o asociada. El dominio SH2 facilita la interacción de VAV1 con quinasas de tirosina aguas arriba, mientras que NSH3 está involucrado en el acoplamiento de moléculas adaptadoras.

Roles fisiológicos de la familia VAV

La función principal de las proteínas VAV es catalizar el intercambio de nucleótidos de las RHO GTPasas, favoreciendo su transición de una conformación inactiva (unida a GDP) a una conformación activa (unida a GTP). Los principales sustratos de las proteínas VAV incluyen RAC1 y, en menor medida, RHOA. A través de la activación de RAC1, las proteínas VAV desencadenan señalización aguas abajo que involucra quinasas efectoras como PAK y JNK, y la estimulación de cascadas de transcripción reguladas por F-actina, como AP1, así como el factor de respuesta sérico (SRF).

A pesar de su alta homología estructural, los tres miembros de VAV no son completamente redundantes desde el punto de vista funcional. La expresión diferencial de las proteínas VAV da cuenta de funciones específicas de tejido en humanos. VAV1 se expresa principalmente en células hematopoyéticas, donde activa RAC1 en respuesta a la transducción de señales tras la activación de células T. La respuesta inmune está en el centro de las funciones de VAV1, incluyendo la respuesta al antígeno en células T, la fagocitosis en macrófagos y la producción de superóxido en neutrófilos. En contraste, los miembros VAV2 y VAV3 están más ampliamente expresados en diferentes tejidos y también desempeñan roles importantes en el desarrollo y la función celular. VAV2 y VAV3 se han estudiado en los sistemas cardiovascular y nervioso, donde desempeñan roles importantes en la angiogénesis y el desarrollo, respectivamente.

Desde una perspectiva estructural, las funciones de las proteínas VAV se pueden clasificar en funciones dependientes e independientes de GEF, realizadas a través de diferentes dominios proteicos. Las funciones de adaptador no catalíticas de VAV1 están mediadas principalmente por los dominios CH y SH de la proteína. El dominio CH de VAV1 promueve la interacción con la fosfolipasa C1 (PLC1), un evento que permite la activación descendente del factor nuclear de células T activadas (NFAT) en linfocitos. Esta vía es esencial para la activación de células T, la producción de citoquinas y la diferenciación de células T foliculares cooperadoras. El dominio CSH3 de VAV1 realiza funciones de andamiaje con una variedad de proteínas, entre ellas, la ligasa de ubiquitina E3 CBL protooncogén B (CBL-B). Esta interacción es importante para amortiguar los niveles del dominio intracelular activo de NOTCH1 (ICN1) en células T. Además, VAV2 también se ha caracterizado en el sistema inmunitario, donde desempeña funciones similares a VAV1 aunque en diferentes linajes hematopoyéticos. Por ejemplo, VAV2 solo puede estimular NFAT en linfocitos B, pero no en células T, donde VAV1 y VAV3 son más importantes.

Proteínas VAV en procesos patológicos

Dada su expresión en células hematopoyéticas y sus funciones inmunes, no es sorprendente encontrar a VAV1 implicada en diversos trastornos inmunológicos. Los polimorfismos de un solo nucleótido de VAV1 se han relacionado con la severidad de enfermedades como la esclerosis múltiple, la artritis reumatoide o la aterosclerosis. También se ha descrito la implicación de VAV1 en respuestas inmunes como el rechazo de injertos de tejido y la enfermedad de injerto contra huésped.

Además de las disfunciones inmunológicas, VAV1 ha despertado interés en el campo de la oncología desde su descubrimiento en 1989 debido a su actividad oncogénica en ensayos de formación de focos. Las RHO GTPasas desempeñan un papel importante en muchos aspectos de la formación tumoral, a menudo impulsada por la sobreexpresión de GEF o la pérdida de inactivación mediada por GAP. Más recientemente, se ha estudiado el papel de las proteínas de la familia VAV en el cáncer, no solo en términos de expresión, sino también a través de mutaciones oncogénicas. Las mutaciones puntuales de VAV1 o las translocaciones que eliminan interacciones clave de autoinhibición se encuentran frecuentemente en diferentes tipos de tumores, donde estas alteraciones confieren a VAV1 una actividad independiente de la fosforilación que la convierte en oncogénica. Los estudios del genoma del cáncer revelaron en los últimos años que VAV1 presenta mutaciones con frecuencia en varios linfomas no Hodgkin, especialmente en los linfomas de células T periféricas (PTCL), y en menor medida en cáncer de pulmón. La inesperada función de VAV1 como supresor tumoral ha llevado a una caracterización detallada de su dualidad estructural-funcional en los últimos años, lo cual ha sido clave para comprender su patogénesis.

Descubrimiento y desarrollo de fármacos

El diseño y desarrollo de fármacos es un área de investigación importante que ha evolucionado durante siglos. La "revolución ómica", los avances en química computacional y el desarrollo de técnicas de cribado de alto rendimiento (HTS) han cambiado sustancialmente el campo en el último siglo, llevando el área a nuevos límites. A pesar de todos los avances tecnológicos, el desarrollo de fármacos sigue siendo, hasta el día de hoy, un proceso largo y costoso. El descubrimiento de fármacos constituye la primera fase del desarrollo de fármacos, descrita en la sección anterior, y se apoya en áreas de investigación básica. La investigación actual sobre fármacos sigue diferentes estrategias que integran información recopilada de múltiples disciplinas como biología, bioquímica, farmacología, matemáticas y modelado computacional. El descubrimiento de fármacos

comienza con la identificación de una molécula diana, generalmente una proteína. Las estrategias de descubrimiento de fármacos se pueden clasificar en diseño de fármacos basado en HTS y relación estructura-actividad (SAR). Ambas estrategias resultan en la identificación de compuestos candidatos: estructuras químicas que muestran actividad y selectividad en un protocolo de cribado bioquímicamente relevante. Los compuestos candidatos necesitan validación y pueden ser mejorados y caracterizados. En la primera estrategia, los fármacos se diseñan específicamente en torno a la molécula diana, integrando los datos estructurales y funcionales existentes. En la segunda estrategia, se realizan cribados de grandes bibliotecas químicas contra la diana elegida. A menudo, ambas estrategias se combinan. Tanto los resultados de acoplamiento SAR como los candidatos de HTS pueden ser refinados a través de rondas de síntesis química, siguiendo un ciclo de síntesis-prueba-síntesis-prueba, hasta obtener un fármaco con la actividad deseada.

Diseño de fármacos basado en estructura-actividad

Los datos ómicos han contribuido significativamente a la refinación del descubrimiento de fármacos. Es importante destacar que los avances en el campo de la biología estructural han remodelado enormemente el campo de la química medicinal. Los investigadores ahora pueden utilizar información estructural detallada sobre las dianas terapéuticas para redirigir los hits de cribado en compuestos candidatos a fármacos. Conocer la conformación 3D de las proteínas también abrió el camino al análisis computacional, un campo emergente en el descubrimiento de fármacos que permite predecir la unión de pequeñas moléculas a la proteína diana. Esta técnica se conoce acoplamiento molecular o *docking*. El *docking* molecular predice la orientación molecular de cualquier ligando dado dentro de un receptor o bolsillo proteico preestablecido. Las posibles orientaciones del ligando dentro del bolsillo son puntuadas por la fuerza de las interacciones no covalentes, utilizadas como medida de la afinidad teórica de unión. La química computacional podría aliviar la baja eficacia y el alto costo que son desafíos actuales del diseño y descubrimiento de fármacos.

A pesar de los avances en el ámbito de la biología computacional, la inhibición de compuestos químicos estándar aún presenta algunos desafíos y limitaciones. Por ejemplo, las proteínas con sitios activos amplios, cavidades de unión inalcanzables o superficies lisas dificultan el diseño de fármacos químicos y a menudo se consideran “intratables”. Nuevas estrategias bioquímicas han sido diseñadas para la inhibición de proteínas fuera de los compuestos químicos estándar en la última década, que buscan resolver estos desafíos. Los inhibidores basados en péptidos de interacciones proteína-proteína (PPIs) y

la tecnología de degradación dirigida de proteínas (TPD) son dos ejemplos de inhibición alternativa de proteínas que están ganando popularidad.

VAV1 como diana terapéutica

Las pequeñas GTPasas han sido consideradas “indrogables” en la investigación del cáncer porque presentan pocas cavidades estables además de su bolsillo de unión a nucleótidos. Su afinidad de unión subnanomolar a GTP o GDP, junto con la concentración fisiológica micromolar de estos nucleótidos, hace que sea un desafío dirigir pequeñas moléculas que modulen estas proteínas, a diferencia de las quinasas proteicas. Por esta razón, la mayoría de las terapias actuales contra el cáncer dirigidas a GTPasas se centran en inhibirlas a través de sus efectores. Debido a sus importantes implicaciones en el cáncer, la superfamilia RAS ha sido la más estudiada como diana terapéutica en el campo, con más de 50 inhibidores actualmente aprobados para tumores con mutaciones en RAS. Los esfuerzos para dirigir las mutaciones de RAS han sido muy costosos, enfrentándose a menudo a la resistencia a los fármacos y a efectos secundarios. Las estrategias para detener el crecimiento de tumores con mutaciones en RAS en los últimos años a menudo han incluido la dirección de los efectores de RAS, siendo los inhibidores de RAF, MEK o mTOR los ejemplos más comunes. No obstante, la identificación de un bolsillo alostérico críptico en el dominio switch II de los mutantes K-RAS estableció la base para el desarrollo clínico de inhibidores covalentes y selectivos de $KRAS^{G12C}$ que han sido aprobados para el cáncer de pulmón de células no pequeñas.

Para la familia RHO en cáncer, la estrategia más utilizada hasta la fecha para el desarrollo de fármacos es dirigir directamente a las RHO GTPasas y sus efectores. La mayoría de los compuestos que dirigen la activación de RHO están orientados a bloquear la unión de GEF, como los inhibidores de CDC42. Si bien se han desarrollado varios inhibidores para RHO, CDC42 y sus respectivos efectores ROCK y PAK, los inhibidores de RAC1 siguen siendo muy escasos. Esto podría cambiar en los próximos años, especialmente a la luz del reciente cambio de paradigma en el desarrollo de fármacos para K-RAS, un avance que ha demostrado potencial para su aplicación en RHO GTPasas. Además, ha habido un aumento en los esfuerzos para dirigir los reguladores de RHO GTPasas en lugar de sus efectores aguas abajo en los últimos años, abriendo nuevas oportunidades para el desarrollo de fármacos que se desvían de las dianas tradicionales.

Inhibidores actuales contra RHO GEFs

A pesar de sus implicaciones en el desarrollo del cáncer, los inhibidores que dirigen a RHO GEFs siguen estando subrepresentados en el campo del descubrimiento de fármacos. Las tecnologías de HTS han llevado a la identificación de algunos inhibidores de RHO GEF, como aptámeros de ARN para TIAM1, péptidos derivados de aptámeros para TRIO y pequeños inhibidores de moléculas para ARHGEF 12. Sin embargo, pocos inhibidores de RHO GEF se han desarrollado siguiendo enfoques de diseño de fármacos SAR, probablemente debido a las dificultades para identificar bolsillos en las proteínas diana. Por ejemplo, la pequeña molécula IODVA1 se identificó inicialmente en una pantalla de biblioteca virtual dirigida a RASG60A oncogénico, y posteriormente se caracterizó como un inhibidor de VAV3, evidenciando la importancia de establecer bolsillos precisos durante la identificación *in silico* de moléculas candidatas. Finalmente, los esfuerzos de reposicionamiento de fármacos han llevado a la identificación de la molécula inmunosupresora Azatioprina como un inhibidor de VAV1, aunque sus efectos adversos podrían dificultar su uso como agente terapéutico en oncología.

El valor terapéutico de VAV1

Las implicaciones patológicas de las alteraciones en VAV1 reveladas en las últimas décadas evidencian su potencial como diana terapéutica tanto en alteraciones inmunitarias como en cáncer. Los extensos datos de modelos murinos resumidos en la sección anterior han contribuido a elucidar el mecanismo de acción mediante el cual VAV1 está implicado en malignidades humanas, estableciendo las bases para su diseño de fármacos dirigido. Dos grandes obstáculos que solían limitar el diseño de fármacos SAR para VAV1 han sido superados. El primero consiste en avances en la caracterización estructural de proteínas que permiten modelar de manera más precisa el complejo GTPasa-GEF, mejorando el potencial de especificidad del fármaco. El segundo es la elucidación de la dualidad de la proteína VAV en cáncer. La rigurosa caracterización de los mecanismos de regulación de la proteína VAV dependientes e independientes de GEF en los últimos años ha abierto una nueva oportunidad terapéutica, evidenciando las ventajas de inhibir la actividad catalítica de VAV1 mientras se preservan sus funciones de supresor tumoral mediadas por el dominio CSH3. Integrando esta información junto con su perfil mutacional recientemente caracterizado, VAV1 ofrece oportunidades únicas y novedosas para el diseño de fármacos dirigidos que merecen ser exploradas.

CONCLUSIONES

Las principales conclusiones de esta tesis doctoral fueron:

1. Los métodos de cribado in silico e in vitro, junto con enfoques de miméticos peptídicos dirigidos a sitios específicos, han demostrado ser estrategias efectivas para la identificación de inhibidores dirigidos a los sitios autoinhibitorios y alostéricos de VAV1.

2. Se identificaron un total de 15 compuestos que exhiben una actividad inhibitoria significativa sobre la GEF VAV1 mediante modificaciones químicas dirigidas de los hits obtenidos in silico, siendo las sustituciones nitro las que favorecen fuertemente la actividad de los compuestos.

3. Los inhibidores caracterizados han demostrado ser específicos para VAV1 y no para la GEF RAS SOS1.

4. Los inhibidores más prometedores de VAV1 se han validado mediante pruebas preliminares en líneas celulares NIH-3T3 transformadas con VAV1 y en respuestas T celulares dependientes de VAV1 en células Jurkat.