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Tetraspanins: useful multifunction proteins for the possible design and development of small-molecule therapeutic tools

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Teaser: Tetraspanins, a family of membrane multifunction proteins with many physiological and pathological implications, represent relevant targets for the design and development of small-molecule therapeutic tools in cancer, immunologic, and infectious diseases.

Author biographies

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Jean-Michel H. Robert is a professor in medicinal chemistry and drug discovery at the Faculty of Pharmacy and director of research in the Department of Medicinal Chemistry of EA 1155-IICiMed in the Health Research Institute (IRS2) of the University of Nantes. He is co-author of eight patents and 50 articles in the field of drug discovery and leads research based on the development of small molecules acting as potent anti-inflammatory, anticancer and immunomodulatory drugs.

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Tetraspanins constitute a well-conserved superfamily of four-span small membrane proteins (TM4SF), with >30 members in humans, with important roles in numerous mechanisms of cell biology. Moreover, tetraspanins associate with either specific partner proteins or another tetraspanin, generating a network of interactions involved in cell and membrane compartmentalization and having a role in cellular development, proliferation, activation, motility, and membrane fusions. Therefore, tetraspanins are considered regulators of cellular signaling and are often depicted as 'molecular facilitators'. In view of these many physiological functions, it is likely that these molecules are important actors in pathological processes. In this review, we present the main characteristics of this superfamily, providing a more detailed description of some significant representatives and discuss their relevance as potential targets for the design and development of small-molecule therapeutics in different pathologies.

Structural features of the tetraspanin superfamily

The first characterization of a tetraspanin protein (CD63 = ME491) was carried out in 1988 and identification of related proteins led to definitions of the characteristics of the family [1–3]. The human genome encodes 33 tetraspanins, systematically named Tspan1 to Tspan33, but this nomenclature is rarely applied and many synonymous terms are used. Tetraspanins (Figure 1) constitute a family of small membrane proteins with four transmembrane helices, three small intracellular regions, and two extracellular domains of unequal sizes, also called extracellular loops. These extracellular loops contain four to eight cysteine residues (four of which are absolutely conserved). The first, third, and fourth transmembrane domains contain highly conserved polar residues forming strong hydrogen bonds among them (Figure 2). These domains have an important stabilizer role during the

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biosynthesis of tetraspanins and promote associations between tetraspanins or between tetraspanins and other proteins; these interactions are determining steps for the construction of a tetraspanin web comprising tetraspanin-enriched microdomains (TEMs). The N and C termini are intracytoplasmic and contain generally <20 residues. They constitute potential binding sites for proteins, such as protein kinase C (PKC) or phosphatidyl inositol 4-kinase (PI4K) ([4] and references therein; [5–11]). Tetraspanins contains also cytoplasmic site (cysteines) for *S*-acylation by palmitoyl-CoA, with palmitoylation generally requiring a functioning Golgi apparatus. These palmitoylations contribute to membrane targeting and localization into TEMs [4,7,8]. Another modification of tetraspanins involves *N*-glycosylation. *N*-glycosylation sites are mainly located in the ECL2 domain and generally occur at classical AsnXSer/Thr sites [12]. In addition, several tetraspanins (CD81, CD82, CD151, and Tspan6) contain cytoplasmic sites that contribute to their downregulation [13–15].

Tetraspanin functions

Although tetraspanins are widely and abundantly expressed in various tissue types, their biological functions are not yet fully identified (Figure 3). However, over the past 20 years, interest in these molecules has increased and it is now well accepted that tetraspanins, by organizing multimolecular membrane complexes, regulate cell migration, adhesion, fusion and signaling events, as well as having a role in metastasis [16–18]. Given these properties, tetraspanins are a facilitating factor in infection processes with, for example, a facilitating action in viral entry and exit [19]. An essential feature of tetraspanins is their ability to establish associations with a variety of partner proteins to constitute a tetraspanin web. Thus, tetraspanins associate not only with other tetraspanins, but also associate with members of other families [e.g. immunoglobulins (Ig), integrins, growth factors, and signaling enzymes] and assembly of the tetraspanin web is based on three levels of interaction [5,6]. Therefore, it was suggested that the main role of tetraspanins is to organize other proteins to facilitate signal transduction. Different types of protein have been identified for their interaction with tetraspanins and range from membrane receptors to adhesion or signal transduction molecules. Whereas some protein–protein interactions can be specific to a given tetraspanin, it appears that multiple different combinations exist between tetraspanins and associated proteins determining a role for tetraspanins as co-stimulatory molecules [18].

Tetraspanins in adhesion signaling

The first family of proteins interacting with tetraspanins are integrins, which are a large group of cell-surface αβ heterodimers acting as receptor for the extracellular matrix. Generally, one α integrin can bind to one or more types of tetraspanins, but the β chain contribute also to the interaction. First examples are the association of CD9 with αIIβ3 integrin in stimulated platelets and the stoichiometric interaction between CD151 and integrin α3β1 [10,11,20–22]. The main integrin associated are those containing the β1 chain, mostly combined with α3, α4 or α6 and less frequently α2 and α5. The tetraspanins studied initially were CD9, CD81, CD82, and CD151, but more recent research provides a broader overview of tetraspanin–integrin interactions ([18] and references therein, [23]). The resulting complexes have an important role in various cellular processes, including adhesion, morphology, motility, fusion, angiogenesis, and synapse formation, as well as tumor progression and invasion. Furthermore, various tetraspanins (e.g., CD9, CD63, CD81, CD82, and CD151) interact with the phosphorylated form of focal adhesion kinase (FAK), which is a cytosolic enzymatic protein, implicating tetraspanins in FAK regulation; recent research demonstrates that CD9 and CD151/FAK interactions are involved in lymphangiogenesis and contribute to T cell activation and interleukin (IL)-2 immune signaling [24–26]. CD151 also regulates the activation of various other signaling molecules, including Akt, Src, paxillin and Rho family GTPases with a cellular context-dependent activating action [27–29].

Tetraspanins and receptor signaling

Growth factors as well as other membrane receptors are also a major group of interacting proteins. Since the functional regulation of transforming growth factor β (TGFβ) and heparin-binding EGF-like growth factor (HBEGF) by CD9 were reported, further tetraspanins (CD9, CD82, and CD151) have been identified to interact with various tyrosine kinase receptors, including TGFβ [30,31], epithelial growth factor (EGFR) [32–34], Erb-B2 receptor tyrosine kinase 2 (ErbB2) [33–36], kinase vascular endothelial growth factor (VEGFR) [37], tyrosine-protein kinase Met (c-Met), [38,39] and stem cell factor receptor or tyrosine-protein Kit (c-Kit; CD117) [40]. A disintegrin and metalloproteases (ADAMs) constitute a family of transmembrane and secreted proteins belong to the zinc protease superfamily and are involved in the regulation of various cellular processes, including signaling, adhesion, migration, and proliferation. These proteins are characterized by the presence of both metalloprotease and integrin receptor binding, and a cytoplasmic domain constituting a binding site for various signal transducing proteins [41]. Therefore, ADAMs have also been identified as major partner proteins of tetraspanins, with ADAM–tetraspanin associations occurring in many pathologies, such as neurological diseases, immunological and inflammatory disorders, infectious diseases, and cancer. TspanC8, Tspan15, CD9, CD81, CD82, and CD98, among others, are also involved in the regulatory mechanisms of ADAM2 [42], ADAM3 [42,43], ADAM10 [42,44–46], and ADAM17 [44,47]. The regulation of G-protein-coupled receptors (GPCRs) by tetraspanins in humans has also been explored and, for example, CD9 and CD81 have been identified as acting binding partners of Gpr56. The functional expression of dopamine D2 receptor is also under Tspan7 regulation [48,49]

Tetraspanins and intracellular signaling

Given that tetraspanins affect the properties of membrane proteins, they also regulate cytoplasmic signaling proteins and TEMs serve as a potential membrane recruitment site. However, the mechanisms of the interactions of tetraspanins, via their N- and C-termini, with intracellular signaling pathways are still not fully understood, perhaps because tetraspanin signaling complexes form and act quickly and dynamically to coordinate intracellular processes [50]. The most significant and well-studied examples of these interactions are the associations of PI4K and PKC with several tetraspanins [10,11,22,51–53]; the recruitment of the intracellular components of the GPCR complex by CD9, CD81, CD151, Tspan3, and Tspan8 [26,54–56]; and Rho-GTPase/CD82 and CD37 interactions that link the cytoskeleton [53,57–60]. Another example of intracellular function attributed to tetraspanins is the protection of β-catenin from degradation. β-catenin is a dual-function protein involved in the regulation of cellular processes including adhesion, cellular signaling, and protein transcription, as well as acting as a co-activator in the Wnt pathway [61]. Degradation of β-catenin occurs via phosphorylation performed by glycogen synthase kinase 3α and β (GSK3α and GSK3β). Mutations and/or overexpression of the β-catenin/Wnt pathway are associated with many pathological conditions, including malignant disease [62]. Many members of the tetraspanin family, including CD9, CD63, CD82, CD151 and Tspan12, are implicated in regulating tumor growth and development in the context of β-catenin/Wnt signaling [63–67].

Given their number and functional organization in networks, the role of tetraspanins in physiological and pathological conditions is not easy to investigate and some remain poorly known. Tetraspanins generally act through associations with other tetraspanins and with partner proteins, making them more complex to study and to understand the mechanisms in which they are involved. Moreover, depending on these protein–protein interactions, the same tetraspanin can have both a promoter and inhibitor effect on cellular signaling, with adverse consequences for their therapeutic use. For example, in malignancies, they constitute useful biomarkers to evaluate the progression and aggressiveness of a tumor, revealing its poor prognosis. In other cases, the expression or overexpression of these molecules might be a favorable element for targeted therapy [63–66]. Despite these difficulties, here we discuss some of the most studied tetraspanins, giving an overview of their properties under physiological conditions to understand their therapeutic potential (Figures 4 and 5).

CD9/Tspan29

CD9 is a 24–27-kDa cell-surface protein of 228 amino acids. It contains four small highly conserved hydrophobic domains , two short N- and C- terminal cytoplasmic domains (11 and seven amino acids, respectively) and a small intracellular loop (four amino acids). The major part of the protein constitutes two extracellular loops, one small (20 amino acids) and one large (83 amino acids). Four well-conserved cysteine residues generate two disulfide bonds and stabilize the large extracellular loop [68,69]. The CD9 protein sequence is well conserved between species (e.g., 90% among humans, mice and rats) and shares many homologies with other tetraspanins, particularly in the transmembrane domains. The human CD9 protein was first crystallized by Umeda *et al.* after purification by metal-affinity and size exclusion chromatography [70].

CD9, first identified as a lymphohemopoietic marker, was later implicated in many other cell processes, including cell motility, heparin-binding epidermal growth factor activity, neurite outgrowth, and myotube formation and maintenance, with a varied tissue distribution (e.g., platelets; early, activated, and differentiating B cells; dendritic cells (DCs); brain and peripheral nerves; kidney; liver; skeletal and smooth muscle) [12,71–74]. These interactions link CD9 to numerous endogenous pathologies, including inflammation, immune diseases, tumor progression, metastasis and chemoresistance [18,75–80].

Given that CD9, as well as other tetraspanins, are involved in various membrane-associated functions, including adhesion, fusion, and trafficking, they constitute gateways for intracellular bacterial, viral, fungal, and parasitic infections [82]. CD9 is one of the main contributing factors for invasion by enveloped viruses by promoting membrane fusion. For example, in Middle East Respiratory Syndrome Coronavirus (MERS-CoV), the CD9-dependant linkage of dipeptidyl peptidase 4 (DPP4) to type II transmembrane protease serine subtype 2 (TMPRSS2) facilitates a robust and early membrane fusion and infection of target cells [19,83]. Likewise, fusion and exit of the influenza A virus (IAV) involves CD9- (but also CD63- and CD81-) dependent processes [84]. In addition, studies of HIV-1 infection mechanisms indicate that CD9 (and also CD81) have an important role in membrane fusion induced by the virus envelope [85]. Finally, CD9 characterizes important populations of cells with regulatory properties involved in cell homeostasis and pathological conditions [75]. CD9 expression was recently shown to define a new population of IL-10-secreting regulatory B cells (Bregs), which effectively inhibit allergic airway inflammation in a murine model of allergic asthma [86]. The role of $CD9^+$ Bregs was further corroborated by the finding that patients with severe asthma have significantly lower percentages of $CD9^+$ Breg compared with healthy volunteers [75]. Finally, CD9 and other tetraspanins, such as CD63 and CD81, are important surface markers of exosomes, the smallest and most studied form of extracellular vesicles [87,88]. Extracellular vesicles are implicated in the pathology of a range of diseases, from cancer to cardiovascular, metabolic, neurological and infectious diseases [88]. Extracellular vesicle-based therapies, particularly using mesenchymal stem cell (MSC)-derived exosomes, are emerging as promising strategies in regenerative medicine and immunomodulatory therapy [89]. Given that CD9 is involved in the biogenesis of exosomes, drugs targeting CD9 could modulate exosome generation and produce therapeutic effects [90].

CD63/Tspan30

CD63, the first characterized tetraspanin, is a highly glycosylated 25-kDa protein, widely but not ubiquitously expressed, present in TEMs, but also and abundantly in late endosomes and lysosomes [91]. CD3 is endocytosed via different pathways, including a clathrin-dependent one with an evident role of caveolae. This tetraspanin also has a major role in the regulation of intracellular transport and localization of proteins, and controls many processes, including cell survival, cell adhesion, spreading, and migration [92]. For example, CD63 (as seen with other members of the tetraspanin family, including CD9, CD81, CD82, and CD151) is present in immature DCs modulating the interactions between immature DCs and their environment by slowing their migratory ability. However, only CD63 has a role in the internalization of complex antigens [93]. Furthermore, macrophages and DCs, both antigen-presenting cells (APCs), initiate immune responses by presenting antigens in the context of major histocompatibility complex II (MHCII) to $CD4^+$ T cells, and CD63 might chaperone MHCII molecules through the endosomal system [93]. Another example of the involvement of CD63 concerns the control of allergic processes. Mast cell (MC) activation leads to the release of high-affinity IgE receptor (FcεRI)-dependent mediators involved in immediate forms of allergic reactions. Investigations of the role of CD63 in MC activation showed that this tetraspanin is required for IgE-mediated degranulation and anaphylaxis, identifying it as an important component of allergic inflammation [94]. Finally, CD63 might be involved in the recycling of the H,K-ATPase pump between the plasma membrane and intracellular storage compartments in gastric parietal cells [91].

Originally identified in the surface of activated platelets and in early-stage human melanoma cells, CD63 involvement in tumor pathologies also has been investigated and it is described as a pro-metastatic factor *via* β-catenin stabilization *in vitro* in different metastatic tumor cell lines [1,37,95]. During malignant melanoma progression, plasma levels of CD63 are higher than in healthy controls. This suggests the use of CD63 as a biomarker for melanoma [96]; the complex of CD63 with tissue metalloprotease inhibitor protein-1 (TIMP1) and β-integrin confers anoikis resistance to melanoma [97]. CD63 is highly expressed during the early stages of melanoma and decreases in advanced lesions, suggesting it as a suppressor of tumor progression [98]. In many other tumors, a correlation is observed between a decrease in CD63 expression and increase in malignancy and, therefore, a poor prognosis. This is observed, for example, in ovarian cancer, lung adenocarcinoma, breast and colon cancers, and esophageal carcinoma, where low levels of CD63 expression might be involved in tumor progression [92,96,99].

given that tetraspanins are important actors in the initial adhesion and endocytosis of intracellular pathogens, the role of CD63 has been investigated in bacterial and viral infections.

In HIV-1 infections, studies linked CD63 to the trafficking of the cell surface-associated chemokine receptor CXCR4, which facilitates HIV-1 entry into T lymphocytes and macrophages, indicating the crucial role of CD63 in HIV replication and infection [100–102]. Other examples include the involvement of CD63 in the biogenesis of extracellular vesicles during herpes simplex virus (HSV), the cellular entry of human cytomegalovirus (HCMV), the control of postendocytotic trafficking of human papillomavirus (HPV) via a complex with syntenin-1, or the promotion of viral ARN replication in astrovirus infections (HAstV-1) [103–106]. Finally, in bacterial infections, CD63 is involved in *Salmonella* infection of human macrophages [107].

CD81/Tspan28

Tetraspanin CD81 is a 26-kDa surface protein originally identified as the target of an antiproliferative antibody (TAPA-1) that inhibits B cell proliferation [3]. The crystal structure of this protein revealed a cholesterol-binding cavity, suggesting a potential modulating activity of CD81 by cholesterol [108]. As with other tetraspanins, CD81 participates in various cellular processes, including membrane organization, cellular adhesion and fusion, and protein trafficking. CD81 is associated in TEMs with several other tetraspanins, including CD9, CD82, CD151, and Tspan8, and its normal physiological functions have been documented in B cells (where CD81 in complex with CD19, CD21, and Leu13 contributes to B cell activation) and in T cell receptor signaling (as a co-stimulatory molecule of CD3 and regulation of the development of T cells in the thymus), defining this tetraspanin in the immune system as a regulator of immune synapse, receptor clustering and signaling [50,109,110]. Involvement of CD81 in pathological processes is also well documented. For example, in liver tumor cells, CD81 stimulates ERK/MAPKinase activity linked with an increase in CD82-associated type II PI4K activity contributing to activation and tumor cell proliferation [111]. However, CD81 both promotes and suppresses tumor growth and metastasis, and low expression of CD81 in hepatocellular carcinoma confers more metastatic potential, as is also the case in gastric tumors and bladder cancer [112–114]. By contrast, in melanoma cell lines, overexpression of CD81 increases migration, invasion, and metastasis via a CD81-dependent upregulation of matrix metalloproteinase (MT1-MMP) [115].

In hematopoietic malignancies, CD81 has an important role in the proliferation of B cell, nonHodgkin, follicular, diffuse large B cell, Burkitt, mantle cell and splenic marginal zone lymphomas, but not in Hodgkin lymphoma and multiple myeloma (MM) [116].

Given its role in adhesion/fusion processes, CD81 was identified as a key entry receptor for hepatitis C virus (HCV). Five proteins of the CD81 protein network are identified as HCV entry facilitators, including calpain-5 (CAPN5) and the ubiquitin ligase Casitas B-lineage lymphoma proto-oncogene B (CBLB) [117,118]. CD81 is also important for the hepatic maturation of *Plasmodium* spp. [119].

CD82/Tspan27

Also called KAI1, CD82 is a 267-amino acid protein with a molecular weight of 30–90 kDa, which varies because of glycosylation and with tissue and cell type [120]. Palmitoylation is required for its biological functions; in the transmembrane domains, the polar residues Asn, Gln and Glu are not essential for glycosylation and dimerization but are needed for CD82 conformational stability and for interactions with CD9 and CD151 [121]. CD84 was demonstrated to be a required for Toll-like receptor 9 [TLR9, an endosomal innate immune receptor binding unmethylated cytosine-phosphate-guanine (CpG) motifs in viral, bacterial and fungal DNA] signaling and to modulate TLR9-dependent NF-κB nuclear translocation [122]. CD82 associates also with other transmembrane proteins, including integrins, growth factors receptors, Ig superfamily members, as well as intracellular proteins. Located in cellular membranes, endosomes, lysosomes, or exosomes, CD82 is ubiquitously expressed in human tissues: B and T cells, natural killer (NK) cells, monocytes, platelets, and various nonhematopoietic cells [120]. Investigations of CD82 functions revealed that it regulates cell aggregation, cell motility, apoptosis, and cancer metastasis [123]. Moreover, CD82 controls the association of cholesterol-dependent microdomains with the actin cytoskeleton in lymphocytes [124].

In infectious diseases, CD82 has a role in the dynamic intracellular trafficking by specific recruitment to fungal (*Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*) and bacterial (*Staphylococcus aureus*) phagosomes before fusion with lysosomes [125]. In infections with human T cell leukemia virus type 1 (HTLV-1), CD82 interacts with virus envelope proteins and inhibits cell–cell fusion and virus transmission [126]. In HIV-1, accessory viral proteins (Vpu and Nef) control tetraspanin (CD81 and CD82) expression and T lymphocytes from patients with HIV-1 showed a reduced expression of CD82; this downregulation appears to be essential to virus spread [84,127].

In malignancies, CD82 is a tumor metastasis and cell migration suppressor in prostate cancer; similar data were observed in many malignant solid cancer cell types, including bladder cancer, breast cancer, pancreatic cancer, and hepatocarcinoma. Studies in renal cell carcinoma showed that the metastasis and invasion-inhibiting action of CD82 occurred via inhibition of TGF-β1/Smad signaling [128]. Although CD82 is widely expressed in various endothelial cells, a study revealed that it restrains pathological angiogenesis by perturbation of CD82-ganglioside-CD44 signaling [129]. Thus, CD82 could be considered a promising biomarker for predicting the prognosis of patients with malignant pathologies [130].

CD151/Tspan24

Tetraspanin 24, generally called CD151, is a 28-kDa and 253-amino acid four-transmembrane glycoprotein, first cloned as a platelet-endothelial cell tetraspan antigen-3 (PETA-3), identified as a platelet cell surface antigen and assigned to human chromosome 11p15.5 [131,132]. As a resident of the tetraspanin-enriched microdomains of many cell types, CD151 has been proposed to cluster laminin-binding integrins α3β1, α6β1 and α6β4, receptors for growth factors HGFR, EGFR and TGF-β1R, and MMP-7, MMP-2, and MMP-9. Thus, CD15 is implicated in many physiological and pathological processes [133].

CD151 regulates the function of laminin-5 binding integrins α3 and α6 and integrin–CD151 microdomains trigger FAK phosphorylation, activating downstream partners of FAK: Src, Cas, Paxillin, and CD151–integrin complex-induced angiogenic effects induces activation of the ERK signaling pathway [7,21–23,27,134–136]. The dissociation of the CD151 and laminin-binding integrin complex facilitates remodeling of epithelial cell interactions with the extracellular matrix and also cell migration [137]. In malignant pathologies, CD151 is an important actor promoting adhesion, proliferation, migration, metastasis, and intertissue cell communication. Its overexpression is often associated with poor prognosis in different types of cancer and various *in vivo* models implicate this tetraspanin as a relevant diagnostic and prognostic marker [138]. For example, CD151–integrin complexes control Met-dependent neoplastic growth [139]; CD151 both impairs heterodimerization of ErbB2/ErbB3 and regulates expression of FGFR2 via a PKC-dependent pathway [140,141] and promotes metastasis by TGF-β1/Smad activation [142] in breast cancer. In addition, CD151 regulates RhoA activation and acts as a regulator of the stability of tumor cell–cell interactions [143]. Thus, CD151 could be a potential marker of poor prognosis for solid tumors, including nonsmall cell lung cancer (NSCLC), glioblastoma, breast cancer, gastric cancer, clear cell renal carcinoma, prostate cancer, colon cancer, and hepatocellular and intrahepatic cholangiocarcinoma [144]. In infectious diseases, CD151 supports HCMV and HPV16 penetration into target cells [145–148]. In addition, CD151 is a crucial host factor of nuclear export signaling in IAV infection [149]. These data hold promise for the development of putative antiviral compounds.

CD231/Tspan7

Tspan7 (also known as CD231) is a 27-kDa cell surface glycoprotein comprising 249 amino acids. It is expressed in various tissues but most strongly in brain (e.g., neurons of frontal cortex, olfactory bulb, and cerebellar cortex). Protein interacting with C kinase 1 (PICK1) is a partner protein involved in internalization and recycling of AMPA receptors (AMPARs) and Tspan7 regulates the association of PICK1 with AMPARs and controls AMPAR trafficking [150–152]. These data suggest that Tspan7 has a key role in the morphological and functional maturation of glutamatergic synapses [153]. Tspan7 was also found to be highly expressed in MM and associated with the molecular chaperone calnexin on the cell surface. *In vitro* and *in vivo* studies revealed that overexpression of Tspan7 significantly reduces tumor development and might be associated with better outcomes for patients with MM [154]. By contrast, in NSCLC, Tspan7 was found to promote migration and proliferation via increased

expression of N-cadherin and promotion of the cell epithelial–mesenchymal transition. Thus, Tspan7 could be a potential target for NSCLC therapy [155].

In viral HIV-1 infection, Tspan7 was identified as a positive regulator of actin nucleation and to be required for DC-mediated transfer of HIV-1 to T cells, providing a way to control HIV-1 transfer to T lymphocytes [156]. In addition, the HSV-1 capsid protein VP26 interacts with Tspan7 and this interaction is required for viral budding and egress from the nucleus [157].

CD37/Tspan26

CD37 is a heavily glycosylated protein of 281 amino acids with a molecular mass of ~40–64 kDa [158]. It is expressed almost exclusively in cells of the immune system, mainly mature B cells, but also in other immune cell types, including T cells, macrophages, monocytes, granulocytes, DCs, and certain leukocyte-derived malignant cells [159]. CD37 is present both on the cell surface and at the level of endosomes and exosomes. In physiological conditions in B cells, CD37 has both prosurvival and proapoptotic roles. It can regulate the membrane distribution of α4β1 integrin followed by activation of the PI3K/Akt pathway and cell survival. However, CD37 can also promote the creation of a complex with molecules including protein tyrosine phosphatase, SHP1, Lyn and Syk tyrosine kinases, and PI3Kγ, leading to Akt inactivation and cell death. In addition, interaction of CD37 with the IL-6 receptor acts as a regulator of suppressor of cytokine 3 (SOCS3) and thereby interferes with activation of the IL-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway [160,161]. In this way, it was shown that, in B cell lymphoma, CD37 could act as a tumor suppressor that directly protects against B cell lymphomagenesis [162]. In infectious disease, recent work revealed that CD37 regulates IgA responses and antifungal immunity against *C. albicans* via a dectin-1/CD37 interaction; dectin-1 recognizes β-glucans in the cell wall of fungi and is involved in cytokine production and killing of fungal pathogens [163].

Discussion: strategies to target tetraspanins

Thus, tetraspanins have emerged as key players in regulating cellular processes, including adhesion, cell migration, angiogenesis, invasion, and metastasis, and are also involved in physiological and pathological conditions; therefore, they appear to be relevant targets to inhibit cell proliferation and metastasis in cancer or the entry, replication, and exit of parasites (especially viruses and fungi) in cell targets. However, despite their number, multiple cellular functions and the fact that some have been described since the 1990s, their clinical potential still needs to realized.

The mains routes of tetraspanin targeting (Figure 6) developed so far involve the use of monoclonal antibodies (mAbs; Figure 6a), recombinant soluble Large extracellular loop 2 (LEL/EC2; Figure 6b), inhibition of palmitoylation (Figure 6c) or also small molecules interacting with the LEL or the C-terminal cytoplasmic PDZ domains (Figure 6d) [164].

Different uses of mAbs targeting CD9, CD37, CD63, CD81, and CD151 have been reported. For example, in cancer, anti-CD37 mAbs were reported to increase the apoptosis of primary chronic lymphocytic leukemia (CLL) cells; BI 836826, a chimeric mouse–human mAb anti-CD37, entered a Phase I first-in-man trial in chronic lymphocytic leukemia (CLL) in 2019 [165,166]. In addition, 5A6, a mAb targeting CD81, inhibited the growth of B cell lymphoma in a xenograft model as effectively as marketed rituximab [167]. Other examples of antitumor antitetraspanin mAbs (e.g., anti-CD9, VJI/10, VJI/20, GR2110, and PAINS-13; and anti-CD151, 1A5) have been reported to induce apoptosis, reduce growth or inhibit invasion, migration and metastasis properties in various human solid cancers, including colon, gastric, prostate and lung cancers [168,169]. In viral infectious diseases, mAbs targeting CD151 have shown promising results as prophylactic agents against IAV [170]. Identification of CD9 as a receptor for coronaviruses [MERS-CoV and systemic acquired respiratory syndrome (SARS)-CoV] and a selective agent in the evolution of their virulence justifies investigations in the use of anti-CD9 antibodies as therapeutic agents against Coronavirus 2019 (COIVD-19) [83,171]. Recombinant soluble LEL/EC2 has been used to study and understand the mechanisms of action of different tetraspanins in various physiological and pathological processes. Soluble LELs from CD9, CD63, CD81, and CD151 potently inhibit CCR5-tropic HIV-1 macrophage infection [172], but, to our knowledge, there has been no therapeutic development of this strategy.

Palmitoylation is crucial for assembly of the tetraspanin web, and it might be that inhibition of this process leads to disorganization of these protein–protein networks, inhibiting their functions. Compounds such as the Golgi-disrupting agent Brefeldin A inhibit palmitoylation of CD9 and CD151 [173], but, again, there is currently no therapeutic application being developed. The development of small molecules as 'blockers' of tetraspanin functions is almost nonexistent and the few studies available concern virological applications (against HCV). Drug discovery research was carried out based on the inhibiting activity of terfenadine (a well-known H1-antagonist, Figure 7) on the human CD81-LEL-Receptor HCV-E2 interaction [174]. This compound was identified in a biological screening to be a moderate inhibitor (27% inhibition, 50 μM); structural optimization, carried out via synthesis of >60 derivatives, led to the identification of compound **1** as a lead compound with 69% inhibition at 50 μM. In the meantime, benzyl salicylate was identified as a moderate inhibitor of CD81/HCV-E2 (25% inhibition, 50 μM) and heterocyclic substituted benzoic acids analogues were synthesized; unfortunately, these compounds showed no increased inhibition compared with benzyl salicylate [175,176]. Another study of the structural basis of interactions of CD81-LEL with benzyl salicylate and fexofenadine (a nontoxic derivative of terfenadine) showed that the binding pockets of these two inhibitors overlap the HCV and membrane interaction sites, presenting challenges for structure-based design and development of therapeutics as anti-HCV drugs [177]. A different approach involving

the synthesis of *bis*-imidazole-derived small molecules designed to mimic the space and hydrophobic features of the solvent-exposed face on helix D region of CD81, which mediates binding to HCV-E2, was proposed. In this work, hundreds of compounds were evaluated, providing the proof that CD81-based mimics (compounds **2**, **3** and **4**, Figure 8) can reversibly disrupt CD81–HCV-E2 binding without short-term effects on the cellular expression of CD81 and other tetraspanins or disruption of TEM assembly [178].

Concluding remarks

Despite the abundance of tetraspanins in humans (33 identified) and the many implications of these proteins (and corresponding TEMs) in physiological and pathological processes, there are few examples of *in vivo* targeting of tetraspanins. These biological functions are widely studied and suggest tetraspanins as significant tools, not only as markers in different diseases (infectious diseases, inflammation, immune diseases, and liquid and solid tumors) but also as therapeutic tools. However, the main difficulty in tetraspanin targeting results from their widespread distribution on numerous cell and tissues types, which could be an obstacle to the development of new drugs. In this context, and because of the greater ease in their design and synthesis, the development of small molecules targeting the many protein–protein interactions in which tetraspanins are involved should represent an interesting opportunity to target various pathologies.

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Figure 1. Phylogenetic tree for human tetraspanins. Common names are given in bold. In black, Tetraspanins in black typically contain six cysteines. For the remainder, the number of cysteines is indicated by the colour: blue, four cysteines; purple, seven cysteines; and green, eight cysteines.

Figure 2. General structure of animal tetraspanins. SEL and LEL/EC represent the two extracellular loops; EC1 is the small loop (20–28 amino acids) and EC2 the large loop (76–131 amino acids). ICL is a small intracellular loop of four amino acids. GS indicates the glycosylation sites, PS, the palmitoylation sites and Ub the

regions of ubiquitination. Green lines (SS) represent disulfide bonds between two cysteines of the EC2 loop. The three green oval zones indicate transmembrane polar residues.

Figure 3. Tetraspanins and cellular signaling. An overview of the main interactions of tetraspanins with different actors in cellular signaling. In the case of G-protein-coupled receptors (GPCRs), these are membrane receptors interacting with extracellular agonists and, thus, are related to receptor signaling; however, specific tetraspanin/G-protein interactions are related to intracellular signaling.

Figure 4. Tetraspanins (Tspan), members of the transmembrane 4 superfamily (TM4SF). Figure details common names and alternative names: A15, cell surface glycoprotein A15; DRAP-27, diphtheria toxin-associated protein-27; KAI1, metastasis suppressor Kangai-1; LAMP3, lysosome-associated membrane protein 3; LIMP1, lysosomal integral membrane protein 1; ME491, melanoma-associated antigen 491; MEL1, melanoma antigen 1; MIC3, leukocyte antigen MIC3; MLA1, melanoma-associated antigen 1; MRP-1, reduced motility-related protein-1; OMA81H, ocular melanoma-associated antigen 81H; PETA3, platelet-endothelial tetraspan antigen 3; Plgp40, platelet membrane glycoprotein 40; R2Ag, R2 leukocyte antigen; SFA1, membrane glycoprotein SFA-1; ST6, suppressor of tumorigenicity 6; TALLA1, T-cell acute lymphoblastic leukemia associated antigen; TAPA-1, target of the antiproliferative antibody-1.

Figure 5. Tetraspanins and their major partner proteins. See main text and cited references for additional details.

Figure 6. Strategies for targeting tetraspanins. Different sites are represented; **(a)** use of monoclonal antibodies (mAbs) targeting the large extracellular loop; **(b)** the 'suicide-target' role of soluble recombinant LEL/EC2, for anti-HCV use; **(c)** inhibition of palmitoylation; and **(d)** interactions with cytoplasmic C-terminal tails.

Figure 7. Chemical structures of known small-molecule inhibitors of tetraspanins.

Figure 8. Examples of symmetric *N,N´*-disubstituted imidazole-4,5-dicarboxamide-based mimics of helix D of CD81 synthesized as inhibitors of CD81-HCV-E2 binding.

Figure 1

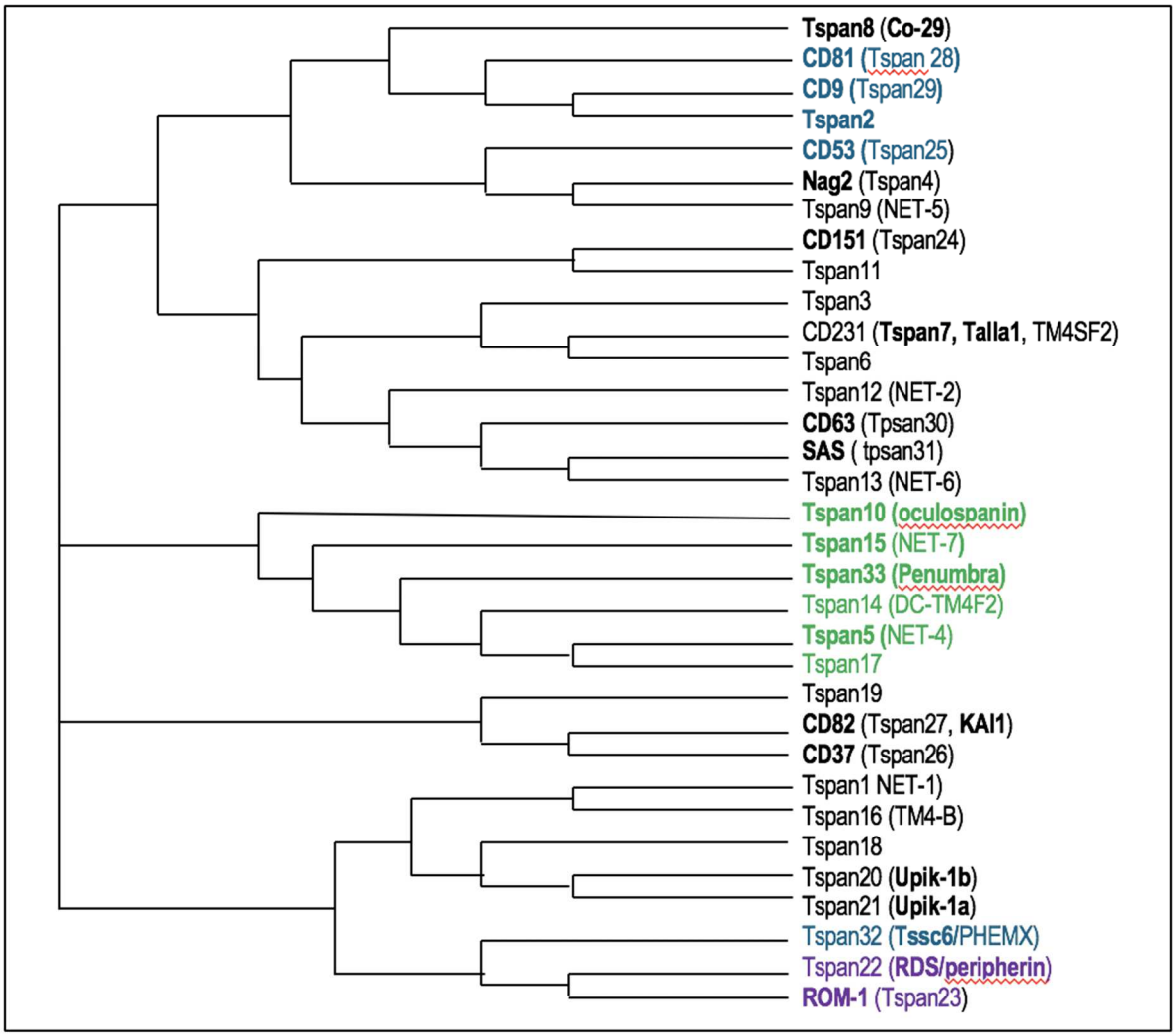

Tspan8 (Co-29)
CD81 (Tspan 28)
CD9 (Tspan29)
Tspan2
CD53 (Tspan25)
Nag2 (Tspan4)
Tspan9 (NET-5)
CD151 (Tspan24)
Tspan11
Tspan3
CD231 (Tspan7, Talla1, TM4SF2)
Tspan6
Tspan12 (NET-2)
CD63 (Tpsan30)
SAS (tpsan31)
Tspan13 (NET-6)
Tspan10 (oculospanin)
Tspan15 (NET-7)
Tspan33 (Penumbra)
Tspan14 (DC-TM4F2)
Tspan5 (NET-4)
Tspan17
Tspan19
CD82 (Tspan27, KAI1)
CD37 (Tspan26)
Tspan1 NET-1)
Tspan16 (TM4-B)
Tspan18
Tspan20 (Upik-1b)
Tspan21 (Upik-1a)
Tspan32 (Tssc6/PHEMX)
Tspan22 (RDS/peripherin)
ROM-1 (Tspan23)

Figure 2

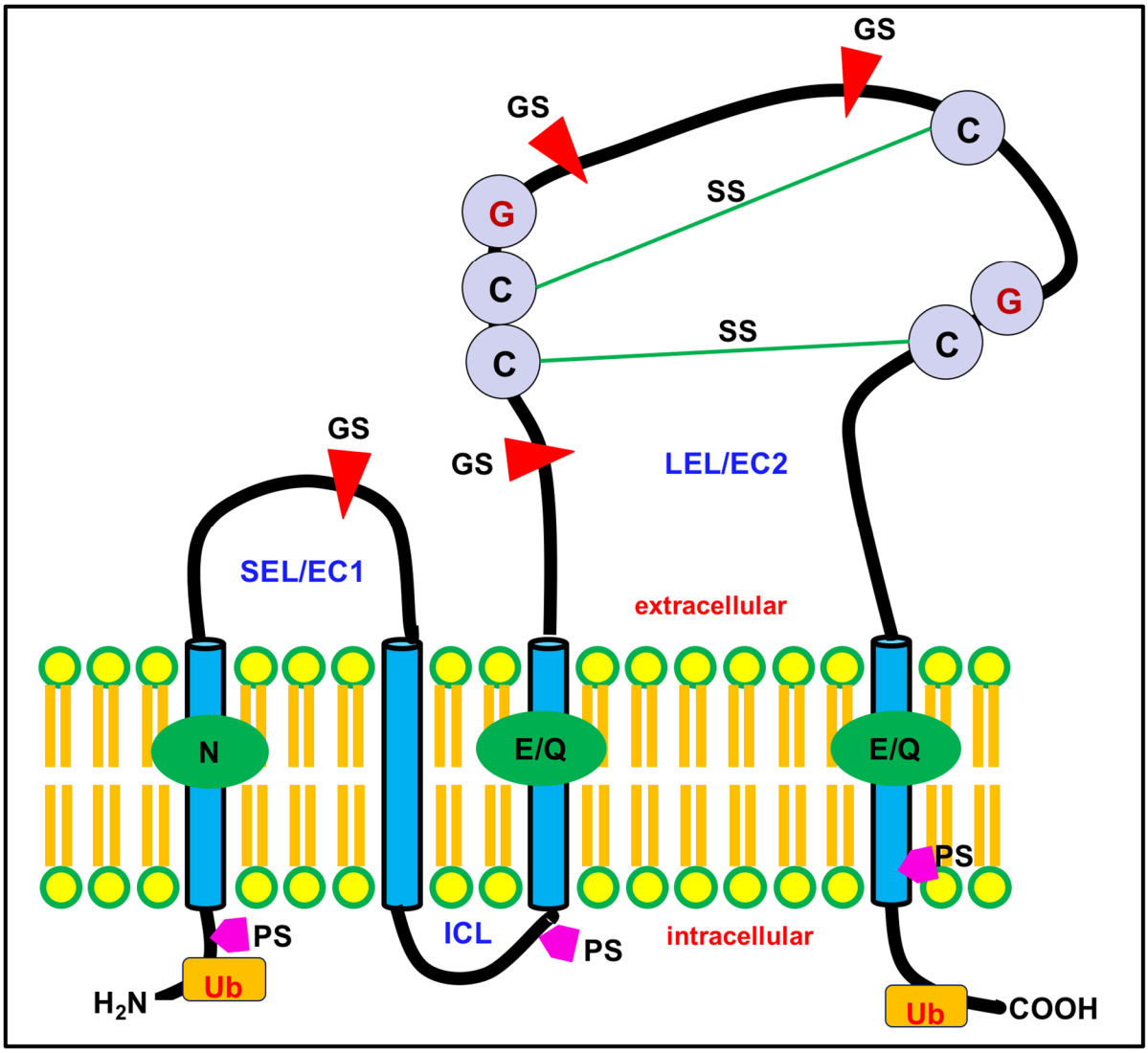

GS
GS
C
SS
G
C
G
SS
C
C
GS
GS
LEL/EC2
SEL/EC1
extracellular
N
E/Q
E/Q
PS
PS
ICL
PS
intracellular
H_2N
Ub
Ub
COOH

Figure 3

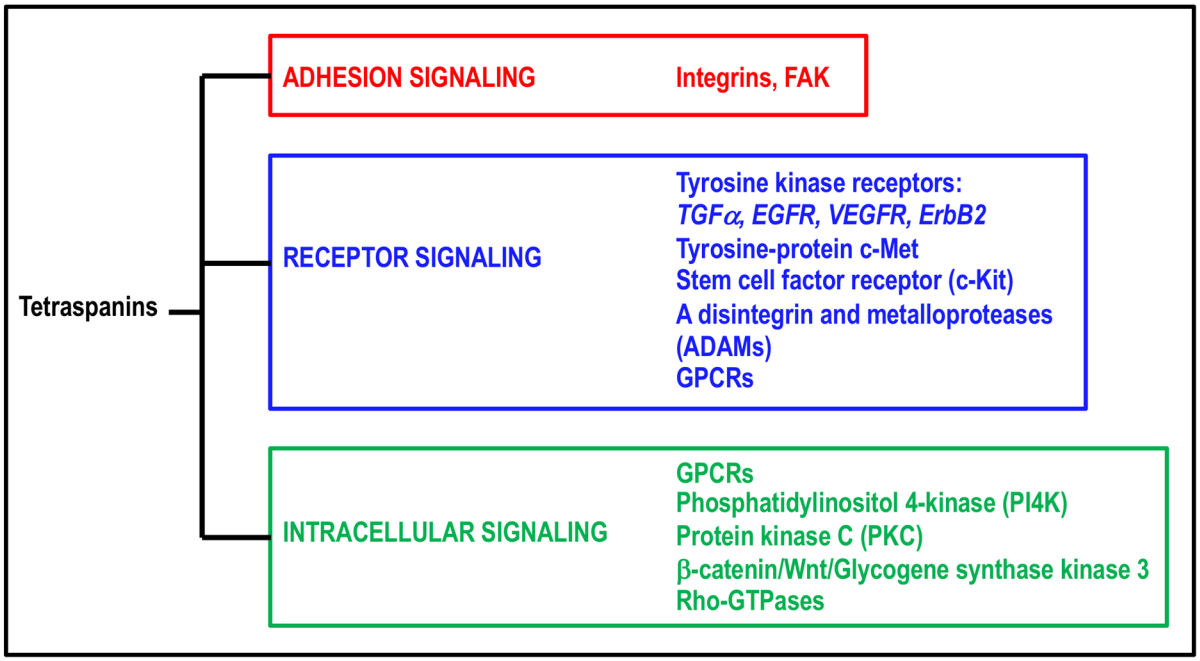

Tetraspanins
ADHESION SIGNALING
Integrins, FAK
RECEPTOR SIGNALING
Tyrosine kinase receptors:
TGFα, EGFR, VEGFR, ErbB2
Tyrosine-protein c-Met
Stem cell factor receptor (c-Kit)
A disintegrin and metalloproteases (ADAMs)
GPCRs
INTRACELLULAR SIGNALING
GPCRs
Phosphatidylinositol 4-kinase (PI4K)
Protein kinase C (PKC)
β-catenin/Wnt/Glycogene synthase kinase 3
Rho-GTPases

Figure 4

Tetraspanin	Tspan	Amino acids	Chromosome	Other names
CD9	29	228	12p13.3	TM4SF2, DRAP-27, MRP-1, MIC3, p24
CD63	30	238	12q12.q13	TM4SF1, MEL1, ME491, granulophysin, LAMP3, OMA81H, MLA1, LIMP1, Pltgp40
CD81	28	236	11p15.5	TM4SF10, TAPA-1
CD82	27	267	11p11.2	TM4SF11, KAI1, ST6, R2 Ag
CD151	24	253	11p15.5	TM4SF32, PETA3, SFA1, gp27,
CD231	7	249	Xp11.4	TM4SF27, A15, TALLA1, TM4SF-2b
CD37	26	281	19q13.3	

Figure 5

Tetraspanin	Major Partner proteins
CD9	$\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrin, PI4K type II, MHC II, IgM, CD3, CD4, CD5
CD63	PI4K type II, MHC II,
CD81	$\alpha 4\beta 1$ and $\alpha 6\beta 1$ integrin, PI4K type II, MHC I and II, IgM, CD3, CD4, CD8, CD19, CD21, CD20, CD225
CD82	$\alpha 4\beta 1$ and $\alpha 6\beta 1$ integrin, MHC I and II,
CD151	$\alpha 6\beta 1$ integrin
CD231	PICK-1, Calexin
CD37	$\alpha 6\beta 1$ integrin, Dectin-1, IL-6R, PI3Kγ

Figure 6

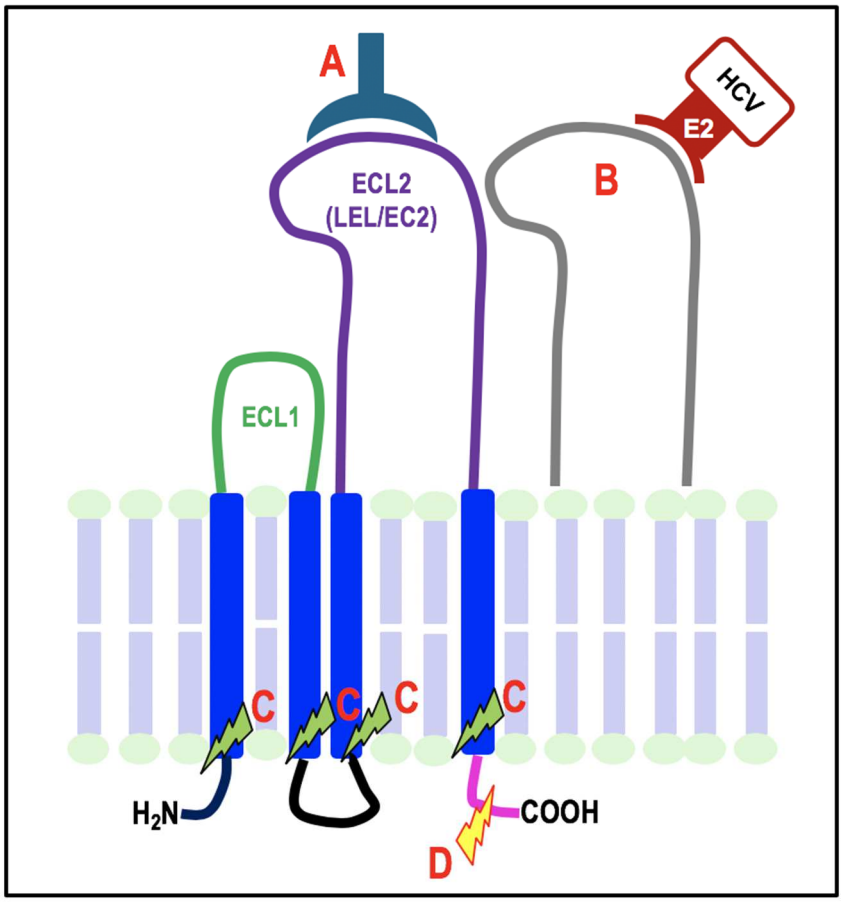


Figure 7

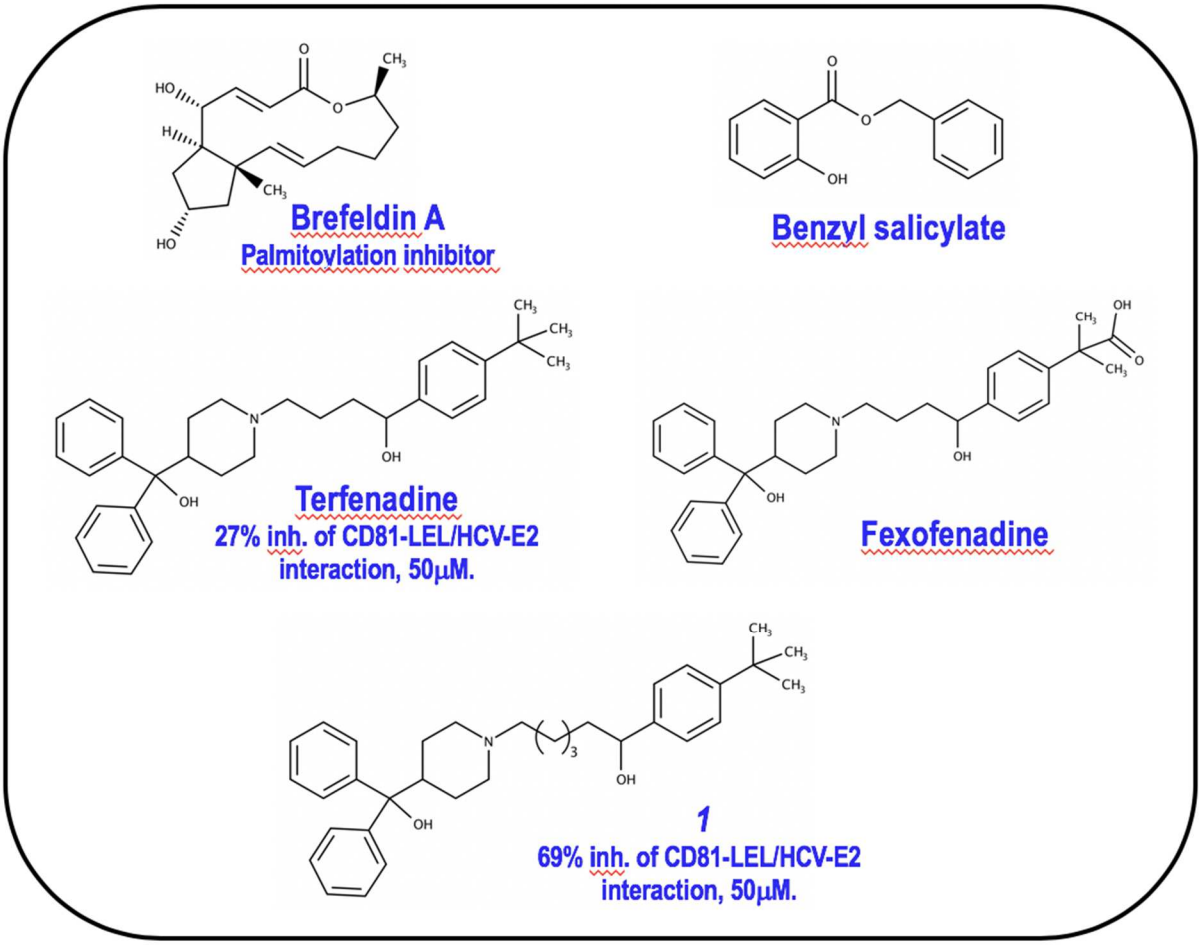
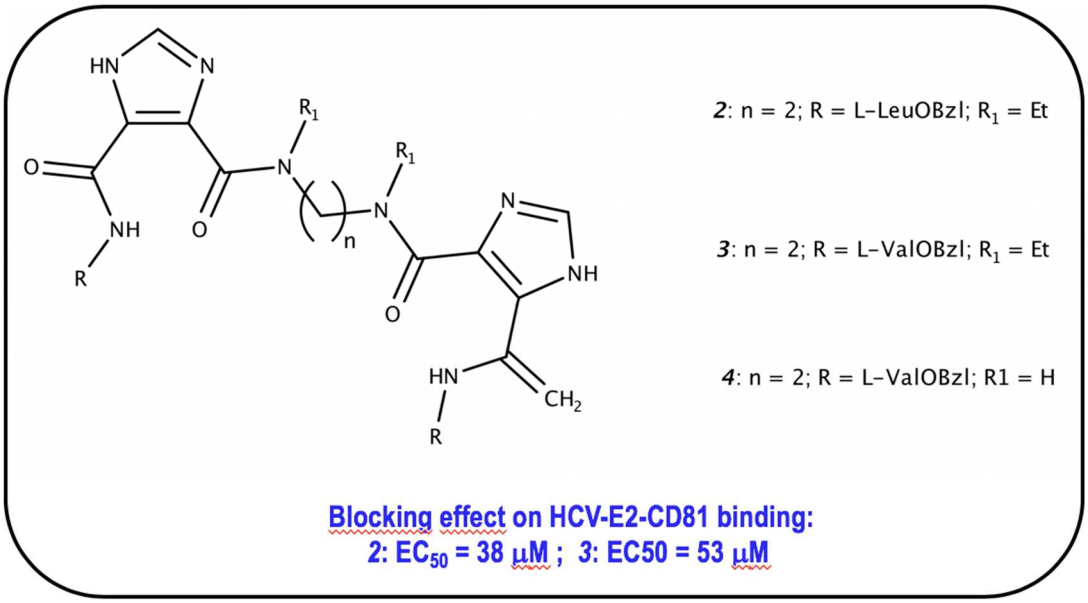

Brefeldin A
Palmitoylation inhibitor
Benzyl salicylate
Terfenadine
27% inh. of CD81-LEL/HCV-E2
interaction, 50μM.
Fexofenadine
1
69% inh. of CD81-LEL/HCV-E2
interaction, 50μM.

Figure 8


HN
N
O
NH
R
O
R1
N
n
R1
N
O
N
NH
HN
CH2
R
2: n = 2; R = L-LeuOBzl; R1 = Et
3: n = 2; R = L-ValOBzl; R1 = Et
4: n = 2; R = L-ValOBzl; R1 = H
Blocking effect on HCV-E2-CD81 binding:
2: EC50 = 38 µM ; 3: EC50 = 53 µM