Tetraspanin CD81

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Tetraspanin CD81 plays major roles in cell-cell interactions and the regulation of cellular trafficking. This cholesterol-embarking transmembrane protein is a co-receptor for several viruses, including HCV, HIV-1 and Chikungunya virus, which exploits the large extracellular loop (EC2) for cell entry. CD81 is also an anticancer target implicated in cancer cell proliferation and mobility, and in tumor metastasis.

CD81 cancer drug targeting natural products

1. CD81 Structure and Dynamic Architecture

Tetraspanins generally comprise four transmembrane domains and intracellular N and C termini. CD81 is a classical tetraspanin, with a standard transmembrane portion flanked by intra- and extra-cellular domains (**Figure 1**). The protein functions as a membrane receptor. It can be overexpressed in cultured cells and purified by immune-affinity [1]. At the structural level, the four transmembrane segments (TM1-TM4) of CD81 define a sort of funnel in which a molecule of cholesterol can bind. The funnel is represented by a four-stranded left-handed coiled coil. A large extracellular loop (EC2) caps the funnel, as a lid at the outer membrane leaflet (**Figure 1**). This large loop EC2 (or LEL) interacts with the vicinal smaller loop EC1 (or SEL) to modulate the conformation of the protein [2]. EC1 contains a small hydrophobic β-strand that packs in a conserved hydrophobic groove of the EC2, which itself is composed of two subdomains, including the δ loop [3][4]. The high-resolution crystal structure of CD81 has confirmed the presence of the two-disulfide bridge in the EC2 domain and a highly-conserved Cys-Cys-Gly motif. The purified full-length human protein has shown a monomeric form, whereas the crystal structure of the extracellular EC2 domain of human CD81 (88 of 236 residues) showed a dimeric assembly [5]. The dimeric form may serve to guide clustering of the different tetraspanin-binding proteins on the cell surface [6]. The organization of CD81 into domains is highly conserved among tetraspanins, notably the transmembrane domain structure, thus facilitating the realization of intra- and inter-molecular interactions and the assembly of the network into the so-called ‘tetraspanin web’ [4][7][8].

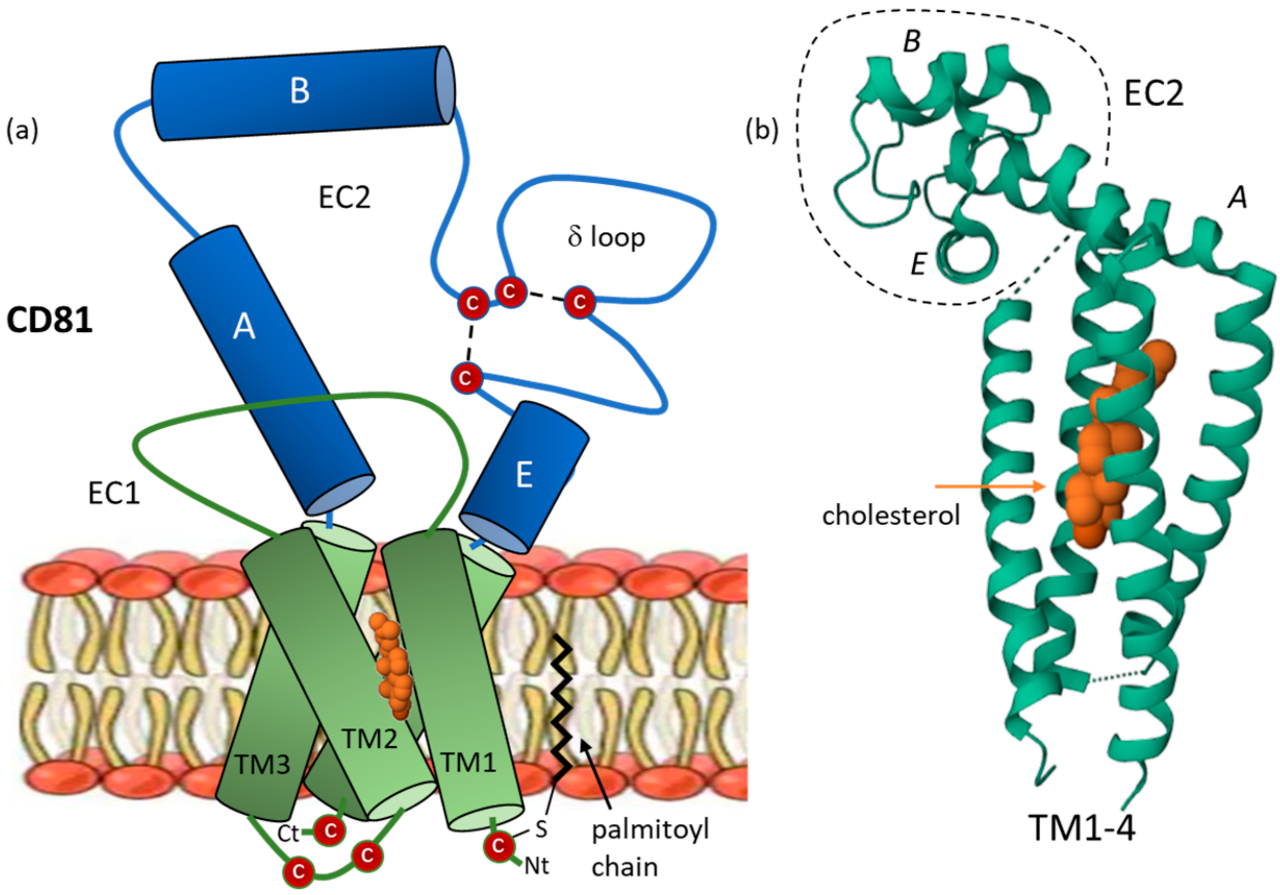

Figure 1. CD81 domain organization and structure. (**a**) Schematic representation of CD81 architecture, with four transmembrane (TM) segments embedded in the membrane bilayer and sequestering a molecule of cholesterol, and two extracellular (EC) loops. The small loop EC1 (or SEL) and the large loop EC2 (or SEL). The disulfur bridges between cysteine residue in EC2 are represented, as well as a palmitoylated cysteine residue contributing the anchoring of the protein into the membrane bilayer. (**b**) A model of CD81, with EC2 in a closed conformation (based in PDB: 5TCX).

A specific feature of CD81 is its capacity to bind cholesterol. There is a well-defined cholesterol-binding pocket which is important to tune the conformation of the protein and exploited by certain viruses to enter into cells. CD81 serves as co-receptor for many viruses, including the hepatitis C virus (HCV), human immunodeficiency virus type 1 (HIV-1), herpes simplex virus 1 (HSV-1), influenza A virus (IAV), Chikungunya virus and a few others [9][10][11][12][13], and occasionally for certain bacteria, such as *Listeria monocytogenes* [14][15], and tropical parasites, such as *Plasmodium yoelii* [16] (**Figure 2**). The role of CD81 in HCV infection has been largely investigated. A potential allosteric mechanism by which cholesterol binding regulates the conformation of CD81 has been identified recently. The cholesterol-free open form of CD81 exhibits a reduced HCV receptor activity compared to the cholesterol-bound closed conformation which presents an enhanced activity for HCV entry. A conformational switch between the two forms operates, delimiting thus a CD81 cholesterol-sensing mechanism [17]. In its closed conformation, the CD81 extracellular loop EC2 disengages from EC1 and changes conformation. This process prevents the binding of CD81 with its main partner CD19 [18].

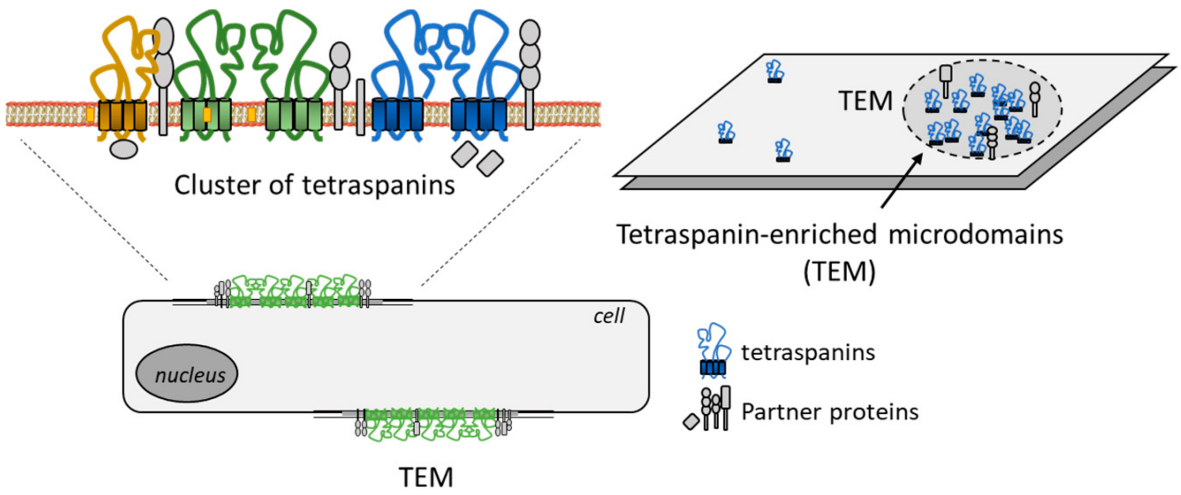

Figure 2. Representation of the clustering of tetraspanins to form microdomains at the plasma membrane, associating divers tetraspanins and partner proteins. The tetraspanin web is a dynamic structural organization at the cell surface, including promiscuous and specific interactions [8].

CD81 is a highly dynamic protein, subject to conformational changes which affect its receptor function and its activity in cells. The tetraspanin protein associates with different protein partners and with cholesterol in the membrane, so as to form protein clusters in membrane microdomains, designated tetraspanin webs or tetraspanin-enriched microdomains (**Figure 2**). These domains correspond to membrane area whereby tetraspanins organize functional higher-order protein complexes, upon interacting with each other and with other transmembrane proteins. CD81 can interact with itself and with other tetraspanins such as CD37, CD53 and CD82 to form individual clusters on the plasma membrane [19]. The sequestering of cholesterol molecules by CD81 within large molecular platforms of proteins induces local conformational changes that perturb the deformability of the membrane [20]. In addition, CD81 is subject to post-transcriptional modifications, notably to a palmitoylation within the intracellular N-terminal segment that is necessary to anchor the protein into lipid rafts [21]. The palmitoylation of cysteine residues of CD81 contributes to the association and anchorage of the protein into the microdomains [4][22]. In contrast, a protein ubiquitination contributes to the removal of the protein from the membrane, through clathrin-mediated endocytosis prior to lysosomal degradation of the protein [23].

2. CD81 Biology, Trafficking and Signaling

Tetraspanins have multiple functions in cells. At the plasma membrane, they promote interactions with other membrane and intracellular proteins and lipids, to shape the organization of membrane domains. They are considered as “molecular facilitators” connecting extracellular and cytoplasmic signaling elements [24]. In this context, CD81 is known to facilitate cells adhesion or fusion in the frame of viral infection [25][26]. The large extracellular loop EC2 (LEL) of CD81 can interact with a variety of proteins, so as to facilitate cellular interaction and capture. Researchers have identified 18 proteins which can interact directly with CD81 (**Table 1**). The tetraspanin certainly interacts with many other proteins, but true CD81-protein interactions have been evidenced experimentally in a limited number of cases. In other situations, interactions have been suggested based on a

colocalization analysis using microscopy for example, but without definitive evidence of direct interaction between CD81 and its colocalization partner. Hereafter, researchers will limit the analysis to the various proteins for which a direct interaction with CD81 has been established (**Figure 3**).

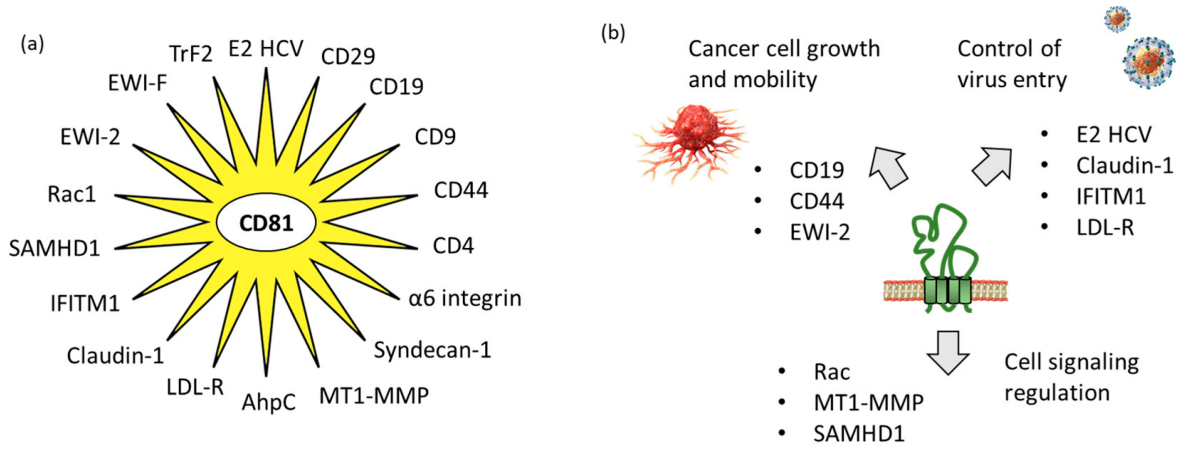

Figure 3. CD81 protein partners. (**a**) Multiple CD81-interacting proteins have been identified, including ten defined with the support of STRING database "https://cn.string-db.org/ (accessed on 1 March 2023)" and other additional proteins discovered through researchers' analysis of the scientific literature. (**b**) The partners include proteins interacting with the extracellular loops of CD81, such as proteins implicated in virus entry into cells and proteins involved in cancer cell growth and mobility, but also proteins interacting with the intracellular portions of CD81 for cell signaling.

Table 1. Proteins interacting with CD81.

Proteins	Types	Interaction and Effects	References
E2 HCV	Viral protein	The transmembrane E2 glycoprotein of HCV utilizes CD81 as a coreceptor for cell entry.	[27][28][29]
Claudin-1	Tight junction protein	The interaction CD81-Claudin1 contributes to HCV infection.	[30]
IFITM1	Tight junction protein	IFITM1 interacts with CD81 to limit HCV entry.	[31][32]
LDL-R	Membrane receptor	Interplay between CD81, LDL-R and PCSK9, to control HCV entry into hepatic cells.	[33][34]
CD19	Membrane receptor	CD81 and CD19 are core subunits of the B cell co-receptor complex. CD81 controls CD19 export activity, via a dynamically regulated process upon B cell activation.	[35]
EWI-2 (IgSF8, PGRL, CD316)	Signaling protein	The CD81/EWI-2 interaction contributes to the tetraspanin web and plays role in cancer cell growth and motility, and in HCV entry.	[36][37][38]

Proteins	Types	Interaction and Effects	References
EWI-F (FPRP, CD9P-1)	Signaling protein	Complexes formed between EWI-F and CD81 (and CD9) play a role in the fusion of myotubes, which are essential elements of muscle architecture.	[39]
CD44	Adhesion molecule	The interaction between CD81 and CD44, through their extracellular regions, promotes tumor cell cluster formation and lung metastasis of triple negative breast cancer.	[40]
α6 integrin	Adhesion molecule	In male germ cells, CD81 interacts with α6 integrin subunit (which forms a dimer with β4 integrin). The complex plays a role in sperm maturation.	[41]
β1 integrin (CD29)	Adhesion molecule	Radiation was found to induce CD29/CD81 complex formation, thereby increasing the cellular uptake of exosomes.	[42]
Rac1	Small GTPase	Interaction of Rac with the C-terminal cytoplasmic portion of CD81 to regulate cell motility. Also has a role in bacterial infection.	[15]
CD4	Cell surface antigen	CD81 interacts with CD4 dimers concentrated in tetraspanin-enriched microdomains.	[43]
CD9	Tetraspanin	Tetraspanins CD9 and CD81 are involved in tetraspanin web formation in sperm. Molecular modelling suggests protein-protein interactions during sperm-egg membrane fusion.	[44]
SAMHD1	Enzyme	CD81 interacts with the deoxynucleoside triphosphate phosphohydrolase SAMHD1 and regulates its expression. The interaction promotes the proteasome-dependent degradation of SAMHD1. It is one of the metabolic regulators of HIV-1 replication.	[45]
MT1-MMP	Enzyme	Several tetraspanins, including CD81, associate with the membrane-type 1 matrix metalloproteinase (MT1-MMP) to regulate its cell surface localization and its function (notably its capacity to activate pro-MMP-2).	[46]
Syndecan-1	Proteoglycan	Knockdown of Syndecan-1 and CD81 inhibits HCV infection, suggesting their cooperative action. A direct interaction between the two proteins has been evidenced (using a proximity ligation assay).	[47]
AhpC	Enzyme	The mycobacterial enzyme alkyl hydroperoxide reductase C (AhpC) interacts with CD81-LEL to promote uptake of the pathogen by host cells.	[48]
TfR2	Membrane receptor	Transferrin receptor 2 (TfR2) is a binding partner for CD81. The interaction triggers RfR2 degradation by the ubiquitin E3 ligase GRAIL.	[49]

One of the prominent binders of CD81-LEL is the E2 envelope glycoprotein which plays a key role in the attachment and entry of the HCV virus into infected cells. CD81 increases the interaction of E2 with membranes and triggers a conformational change in E2 necessary for subsequent membrane fusion [27]. In fact, via E2 the virus utilizes different proteins as co-receptors, in particular, the tight-junction proteins claudin-1 and occludin

together with CD81 and the protein SR-B1 (scavenger receptor class B member 1, encoded by gene *SCARB1*) [50]. SR-B1 is an 82-kDa transmembrane glycoprotein playing an important role in the regulation of cholesterol exchange between cells and high-density lipoproteins. Does CD81 directly interact with SR-B1? There is some evidence for that, notably data indicating that HCV E2 links a soluble form of CD81 and SR-B1 protein together. This physical neighboring could explain why both CD81 and SR-B1 are indispensable factors for HCV infection [51]. Moreover, the physical interaction between CD81 and claudin-1 has been firmly demonstrated. Claudin-1 oligomers associate with CD81 to form complexes playing a role in HCV infection [30][52]. In the same vein, a physical interaction has been evidenced between CD81 and protein IFITM1 (interferon-induced transmembrane 1) which is a hepatocyte tight junction protein implicated in HCV entry. The interaction of IFITM1 with HCV coreceptors CD81 and occludin disrupts the process of viral entry [31][32]. The low-density lipoprotein receptor (LDL-R) is involved also in viral entry. The formation of complexes between CD81, LDL-R and the serine proteinase PCSK9 (proprotein convertase subtilisin kexin type 9) has been observed [33]. PCSK9 enhances the degradation of the LDL-R and modulates liver CD81 levels [34]. However, the targeting of PCSK9 (implicated in cholesterol metabolism) with a mAb (alirocumab) does not affect CD81 and does not modulate HCV entry in hepatic cells [53].

2.2. CD81 Protein Partners Implicated in Tumor Growth and Dissemination

Another essential partner of CD81 is the B-lymphocyte coreceptor CD19, which is a key activator of the PI3K pathway and a prominent tumor-associated antigen. CD19 is a B/plasma cell-lineage marker and an essential target to combat B-cell malignancies, through the design of anti-CD19 chimeric antigen receptor (CAR) T-cell therapies [54]. For example, the CAR T-cell therapy axicabtagene ciloleucel has been recently approved for the treatment of relapsed or refractory follicular lymphoma [55]. Binding of CD19 to CD81 induces opening of the ectodomain and a reorganization of transmembrane helices, resulting in the occlusion of the cholesterol binding pocket [35]. This CD81-CD19 interaction, dynamically regulated upon B cell activation [56], is essential to the correct exposure of CD19 on the surface of B cells. Loss of CD81 expression results in an intracellular accumulation of CD19 [57]. Similarly, a mutation in the *CD81* gene leads to the expression of a truncated protein which does not enable CD19 maturation and cell surface expression [58]. The CD19-complex consisting of CD19, CD81, CD21 (also known as CR2, for complement receptor 2) and CD225 acts as a co-receptor to the B cell receptor (BCR). This complex cannot form properly when CD81 is mutated, causing severe diseases, but fortunately CD81 mutations are extremely rare in humans [59]. CD81 is an essential T cell costimulatory molecule. Its co-stimulation enhances naive T cell activation and largely modulates the activation of chimeric antigen receptors (CAR) [60].

CD81 associates with the two EWI proteins EWI-2 and EWI-F which are also partners of tetraspanin CD9 (**Table 1**). These two interactions are important because they have implications for cancer. EWI-2 (also called PGRL in mouse (prostaglandin regulatory-like protein)) is a regulator of both CD81 and CD9 functions [61], acting as a sequester to prevent the two tetraspanins from providing support to the TGF-β signaling. When EWI-2 binds CD9/CD81, the tetraspanins can no longer support the association of TGF-β receptors 1 and 2 (TβR2-TβR1). This signaling pathway is largely involved in melanoma growth, invasion and metastasis [36][62]. The suppression of CD81 with a shRNA in mesenchymal breast cancer has been shown to reduce primary tumor growth, extravasation

and lung metastasis in vivo [63]. Therefore, the pharmacological blockade of CD81 could be an option to alter the malignant process.

The CD81/EWI-2 interaction has multiple functions, notably acting as a linker of the tetraspanin web to the actin cytoskeleton [64], and this interaction plays roles in different pathologies. CD81/EWI-2 interaction is a regulatory factor for glioblastoma cell growth and motility [37], but also for HCV and HIV infection [38][65]. Notably, the HIV-1 virus uses CD81-lined vesicle structures to infect astrocytes, and then these energy-consuming glial cells support trans-infection of HIV-1 to T-cells [66]. The virus uses CD81 as a rheostat to control different stages of the infection via interaction with different proteins such as EWI-2, but also the deoxynucleoside triphosphate phosphohydrolase SAMHD1 (sterile alpha motif and histidine aspartic acid domain containing protein 1) [45]. Through direct binding to SAMHD1, CD81 regulates the expression of the protein by promoting its proteasome-dependent degradation. This mechanism is implicated in the control of HIV-1 replication [45]. The viral restriction factor SAMHD1 is also considered as an anticancer target. The protein is frequently upregulated in cytarabine (Ara-C)-resistant AML [67]. SAMHD1 inhibitors are being developed for the sensitization of leukemia cells to nucleoside analogue-based therapy [68]. The link between SAMHD1 and CD81 may explain, at least in part, why CD81 is an adverse prognostic marker in AML [69].

The interaction of tetraspanins with the cell surface protein EWI-F (also known as CD9P-1 (CD9 Partner-1) or FPRP (F2alpha prostaglandin receptor regulatory protein)) has been well characterized. EWI-F is an immunoglobulin domain molecule which chiefly interacts with CD9 to form a 2:2 tetrameric arrangement implicated in the formation of tetraspanin-enriched microdomains [70]. In myoblasts, both CD9 or CD81 associate with EWI-F and the complex plays a role in muscle architecture (fusion of myotubes) and muscle regeneration [39]. Here again, the CD9P-1/tetraspanin complex functions as a regulator of cell motility [71]. Interestingly, a truncated form of EWI-F/CD9P-1, designated GS-168AT2, produced in human endothelial cells has been shown to inhibit angiogenesis and cell migration. GS-168AT2 corresponds to the sequence by which CD9P-1 physiologically associates with CD81 [72]. GS-168AT2 binds CD9 and CD81 and displays antitumor activity in vivo, associated with a downregulation of CD9 but not of CD81 [73]. This work suggested that the pharmacological modulation of the tetraspanin web could represent a new anti-angiogenic strategy [74]. The interaction between CD81 and CD9P-1 could also be exploited in the field of parasitic pathologies, because it has been shown that binding to and regulation of CD81 function by CD9P-1 represents a negative regulator of infection by *Plasmodium yoelii* (a malaria pathogen in rodent species) [16]. CD81 is implicated in the uptake of different pathogenic agents. A recent study highlighted the key role of CD81 in the uptake of pathogenic mycobacteria *Mycobacterium abscessus* through interaction with alkyl hydroperoxide reductase C (AhpC), a peroxiredoxin enzyme able to decompose several kinds of hydroperoxides [48]. The targeting of CD81 could be further exploited to combat different infectious diseases.

The hyaluronan-binding protein CD44 (a hyaladherin) is also a binding partner for CD81. A complex formation between these two membrane proteins has been evidenced recently [40]. They interact with each other through their extracellular regions and this recognition facilitates the formation of a tumor cell cluster and lung metastasis of triple negative breast cancer (TNBC). This interaction confirms the key role of CD81 in the migration and invasion

of TNBC cells previously suggested by a proteomic analysis [75]. The invasion of TNBC cell lines can be halted with the use of a specific anti-human CD81 antibody (5A6) [76].

The CD81 interactome includes also the small GTPase Rac and the interaction plays roles in cancer cell motility and exosome formation [77]. The interaction of Rac (Rac1) with the C-terminal cytoplasmic domain of CD81 regulates the GTPase activity [78][79] and the modulation of Rac1 GTPase functions represents a key anticancer mechanism [80]. This tetraspanin-dependent regulation of cell motility raises an opportunity to control cancer cell migration with C81-targeted drugs. In addition, the CD81-Rac interaction plays a regulatory role in the innate and adaptive immunity against bacterial infection [15]. The signaling activity could be blocked with small molecules targeting the intracellular part of CD81 but probably also via targeting the extracellular protein loop.

Finally, researchers can evoke the association of tetraspanins with various integrins to modulate their function. Both tetraspanins CD9 and CD81 are a potential partner of αV integrin, at least in the testicular tissue where they are implicated in sperm development [81][82][83]. These two tetraspanins were also shown to link the cell adhesion molecule JAM-A to αvβ5 integrin and thus to play a role in the regulation of cell motility [84]. CD81 forms a complex with αV/β1 and αV/β5 integrins [85]. Integrins bind to tetraspanins such as CD81 via interaction with the constant region of the EC2 domain [84]. There are other integrins capable of interacting with CD81, such as α3β1 integrin, CD29, and others [42][85][86].

Altogether, researcher' biological network analysis points out 17 protein partners for CD81 (**Table 1**). Most of them interact via the extracellular domain- of the tetraspanin, but in some cases the interaction concerns the intracellular portion, as depicted in **Figure 3**. There are probably many other partners, associated with CD81 itself or with CD81-containing tetraspanin platforms. A proteomic analysis mapped 33 host protein interactions of CD81 in primary human liver and hepatoma cells, including for example protein CAPN5 (calpain-5) and ubiquitin ligase CBLB (Casitas B-lineage lymphoma proto-oncogene B), capable of forming a complex with CD81 and implicated in HCV entry [87]. CD81 presents a dynamic expression profile and the multiplicity of partners is not surprising for a protein implicated in intercellular communication. Therefore, therapeutic molecules interacting with CD81 could serve as interrupters or regulators of different pathways and cellular processes. The next section provides an overview of CD81-binding molecules, large and small.

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