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PDZ Domain in the Engineering and Production of a Saporin Chimeric Toxin as a Tool for Targeting Cancer Cells

Francesco Giansanti,[1] Domenica Sabatini,[1] Maria Rosaria Pennacchio,[1] Stefano Scotti,[1] Francesco Angelucci,[1] Anne-Chloè Dhez,[1] Andrea Antonosante,[1] Annamaria Cimini,[1,3] Antonio Giordano,[2,3]* and Rodolfo Ippoliti[1]**

[1] *Department of Health, Life and Environmental Sciences, University of L'Aquila, I-67100 L'Aquila, Italy*

[2] *Department of Medical, Surgery & Neurosciences, University of Siena, Italy*

[3] *Sbarro Institute for Cancer Research and Molecular Medicine and Center for Biotechnology, Temple University, Philadelphia, Pennsylvania*

ABSTRACT

In this paper we have studied a PDZ protein domain as a possible tool for cellular targeting of the ribosome inactivating protein Saporin, exploiting the ability of PDZ domains to recognize and bind short peptide sequences located at the C-terminus of a cognate protein. We have focused our attention on the PDZ domain from hCASK (Human calcium/calmodulin-dependent serine protein kinase) that binds extracellular CD98 in epithelial cells, being this antigen recognized as a marker for several human tumors and particularly considered a negative prognostic marker for human glioblastoma. We produced recombinant fusions of one or two hCASK-PDZ domains with the ribosome inactivating protein Saporin and assayed them on two human glioblastoma cell lines (GL15 and U87). These constructs proved to be toxic, with increasing activity as a function of the number of PDZ domains, and induce cell death by apoptotic mechanisms in a dose-dependent and/or time dependent manner. J. Cell. Biochem. 116: 1256–1266, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: PDZ DOMAINS; CHIMERIC TOXINS; GLIOBLASTOMA; SAPORIN; hCASK

The search of new targeting systems is crucial for the design of innovative anticancer molecules. In this respect particularly interesting appear to be PDZ domains, found in proteins involved in many cellular processes, from the signal transduction to the transport and particularly in synapse formation in mammals [Kurakin et al., 2007]. The first PDZ domains were discovered as sequence repeats in the primary structures of the post-synaptic density 95 (PSD95), disc-large (Dlg) and zona occludens-1 (ZO-1) proteins [Cho et al., 1992; Sanford et al., 2004]. PDZ domains consist of 80–90 amino acids comprising six beta-strands (betaA to betaF) and two alpha-helices, A and B, compactly arranged into a globular structure. Peptide binding of the ligand takes place in an elongated surface groove as an antiparallel beta-strand interacting with the betaB strand and the B helix [Jemth and Gianni, 2007]. The structure of PDZ domains allows binding to a free carboxylate group at the end of a peptide through a carboxylate-binding loop between the betaA and the betaB strands. They recognize and bind short peptide sequences located at the C-terminus of a protein with K_d in the range 1–50 μM [Gianni et al., 2005, 2006].

To test PDZ domain as possible carriers for toxic molecules, we pointed our attention to the CD98 complex as a target on the surface of cells (also named 4F2, MDU1, 4F2HC, 4T2HC, NACAE), an aminoacidic carrier localized on the cellular surface formed by covalent bonding of the heavy chain CD98hc with different light chains (LAT1, LAT2, y1LAT1, y1LAT2, xCT, and asc-1) [Fenczik et al., 2001; Verrey et al., 2004]. CD98 has a single trans-membrane domain formed by amino acids 82–104, the cytoplasmic portion formed by the amino acids 1–81 and the extracellular domain formed by amino acids 105–529. The human CD98 C-terminus contains a potential binding domain for PDZ, thus suggesting that

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*Correspondence to: Antonio Giordano, Department of Medicine, Surgery & Neurosciences, University of Siena, Viale Mario Bracci 16,53100, Siena, Italy. E-mail: giordano12@unisi.it

**Correspondence to: Rodolfo Ippoliti, Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio snc Coppito, 67100 L'Aquila, Italy. E-mail: rodolfo.ippoliti@univaq.it

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CD98 may be associated with extracellular proteins containing PDZ domains. This region is pointed out as hCD98 and binds the class II PDZ domain in the extracellular space (amino acids 520–529) [Yan et al., 2007, 2008].

The hCASK, serine kinase calcium/calmodulin-dependent of MAGUK family (superfamily of membrane-associated guanylate cyclase domain with enzymatically active guanylate cyclase), is one of the proteins that can bind to the C-terminus of hCD98 in the extracellular space; it has a unique domain that includes the N-terminus with homology to the protein kinase calcium/calmodulin-dependent, followed by three characteristic MAGUK domains (PDZ Class II, domain and SH3 domain GUK) [Harris and Lim, 2001]. hCASK was localized in the basolateral membrane of intestinal epithelial cells [Nguyen et al., 2012]. and was recently demonstrated that hCD98 and hCASK are co-located and co-precipitate together both in vitro and in vivo, and that the bond between the PDZ domain and the CD98 is involved in these interactions [Yan et al., 2007].

CD98 is expressed in many cells types, including renal tubules in the gastrointestinal tract, and polarized epithelial cells, [Rossier et al., 1999; Merlin et al., 2001; Dave et al., 2004] but very high expression of CD98 has been reported in different types of tumors. The light chain LAT1 conjugated with CD98 has also been identified in glioma cells and it is expressed at lower levels in the blood-brain barrier [Yanagida et al., 2001; Takeuchi et al., 2008; Kobayashi et al., 2008; Okubo et al., 2010]. Furthermore several studies indicate the important role of LAT1 and CD98hc in malignant transformation and carcinogenesis so that the complex LAT1-CD98hc may represent a unique target for intervention on cancer. The CD98 is widely expressed in rapid growing cells, where it can also modulate oncogenic transformation and cell fusion. LAT1 was found with high expression levels in rat tumors (*Rattus norvegicus*), such as gliomas (C6), hepatoma (dRLh-84), and hepatocellular carcinoma (FAA-HTC1), while the normal liver does not express LAT1. High levels of LAT1 were also found in other human cancer cell lines derived from stomach, melanoma, and small cell lung [Kanai et al., 1998]. The CD98 is believed to be used in the regulation of amino acid transport and it appears deactivated after the G1 phase, when cells are activated and proliferation is enabled. Instead the complex is up-regulated in tumor cells that are in rapid division and proliferation making within these essential amino acids in order to grow and differentiate [Nawashiro et al., 2002].

For these reasons we decided to verify if PDZ domains may be used to target CD98 in human glioblastoma cells as a model system, and we used as a toxic domain the ribosome-Inactivating Protein (RIP) saporin produced by *Saponaria officinalis* L., a toxin widely studied for the production of immunotoxins (conjugates of an antibody or its fragments and a toxin [Flavell et al., 1991; Flavell 1998; Fabbrini et al., 2003; Anselmo and Mitragotri, 2014]) and targeted toxins [Ippoliti et al., 1996; Giansanti et al., 2010; Lombardi et al., 2010; Cimini et al., 2012].

We designed and produced recombinant chimeric toxins made up of the PDZ-hCASK sequence linked to Saporin S06 as monovalent (hCASK-SAP) or bivalent toxin [containing two PDZ domains $(hCASK)_2SAP$)] and assayed them for their cytotoxicity on human glioblastoma cells lines (GL15 and U87).

hCASK-SAP and $(hCASK)_2$-SAP were toxic towards glioblastoma cells inducing cell death via apoptosis and their action resulted in higher toxicity when co-administered with Saponin, a known enhancer of Saporin toxicity in chimeric constructs [Bachran et al., 2011; Weng et al., 2012]. The results obtained in this work represent the proof of concept, for the first time, that a PDZ-mediated targeting system can be used to deliver toxic molecules to cancer cells, potentially representing an alternative to the use of antibodies or growth factors in the constructions of new anticancer molecules.

MATERIALS AND METHODS

REAGENTS

All the reagents were from Sigma-Aldrich (St. Louis, MA), unless otherwise indicated. All the restriction enzymes, reagents and enzymes for molecular biology were obtained from Fermentas GMBH (St. Leon-Rot, Germany). Saponin SA1641 (purified from an extract obtained from the roots of the plant *Gypsophila paniculata* L. was a kind gift of Prof. H. Fuchs (Charité - Universitätsmedizin Berlin).

BACTERIAL STRAIN AND GROWTH CONDITIONS

For cloning and expression, DH5α and BL21 (DE3) (or Rosetta Gami™ B pLysS(DE3) *Escherichia coli* strains were used, respectively. Rosetta-gami™ host strains are Origami™ derivatives that combine the enhanced disulfide bond formation resulting from trxB/gor mutations with enhanced expression of eukaryotic protein that contain codons rarely used in *E. coli*. These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, and GGA on a compatible chloramphenicol-resistant plasmid. In the Rosetta-Gami™ (DE3) pLysS the rare tRNA genes are present on the same plasmids that carry the T7 lysozyme protective system and *lac* repressor genes and possess Chloramphenicol resistance (all bacterial strain was purchased by Novagen, Merck KGaA, Darmstadt, Germany).

For the growth curve, transformed bacteria were inoculated into LB broth supplemented with the appropriate selective antibiotic (ampicillin and/or kanamicin [50 μg/ml], chloramphenicol [34 μg/ml]) and cultured overnight at 37°C in a New Brunswick G25 incubator. The day after, bacteria were diluted 1:200 into 50 ml of fresh medium and the OD_{600} was checked every hour. For the classical inductions, the overnight culture was always diluted 1:200 in a fresh medium and cultured at 37°C. When the OD_{600} value reached 0.4–0.6 protein expression was induced by adding 1 mM IPTG to 500–1,000 ml of culture. Incubation was continued under vigorous shaking at desired temperature (25, 30 or 37°C) for 4 h. Finally, the cells were centrifuged at 3,000*g* for 15 min and tested for protein expression

GENES AND VECTORS

The synthetic genes was designed based on hCASK gene (**O14936 isoform 1** (CSKP_HUMAN) and SAPSO6 gene P20656 (**RIP6_SAPOF)** (http://www.uniprot.org/uniprot/). pET11a or pET28 vectors (New England Biolabs) were used to perform subcloning of both hCASK-SAP or $(hCASK)_2SAP$ synthetic gene purchased from Life-Thecnolgies. The hCASK gene was kindly provided from

Prof. Dr. Didier Merlin (Emory University Atlanta, GA) into a pcDNA vector and successively extracted by PCR amplification and subcloned in desired vectors.

ESCHERICHIA COLI COMPETENT CELLS AND TRANSFORMATION

Competent cells. *E. coli* (DH5α, BL21, Rosetta Gami™ B pLysS(DE3) chemically competent or electrocompetent cells were prepared using standard protocols [Sambrook et al., 1989].

CELLULAR FRACTIONATION

Induced bacterial pellet was resuspended in 30 mM Tris–HCl pH 8, 2 mM EDTA and 27% sucrose supplemented with lysozyme (2 mg/ml), and incubated for 1 h at +4 °C. The cell suspension was centrifuged at 18,000*g* for 20 min. The supernatant constitutes the periplasmic fraction and the pellet (protoplastic fraction) was further treated in the buffer above described supplemented with 0.1 mM dithiothreitol, proteases inhibitors (pepstatin 1 mg/ml, leupeptin 1 mg/ml, aprotinin 10 mg/ml, and PMSF 15 mg/ml) and 1 mg/ml DNase. This fraction was sonicated using a Vibracell VCX (160 watt), on an ice bath and centrifuged at 18,000*g* for 20 min. The supernatant corresponding to the cytosolic fraction was stored and the pellet of debris was discarded.

SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

The bacterial lysates were analyzed with SDS–polyacrylamide gel electrophoresis (10% or 12% of polyacrilamide) according to the Laemmli method. The bacterial pellet derived from 1 ml of culture was resuspended in 50 μl of reducing sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 4% SDS, and 5% β−mercaptoethanol and Bromophenol blue).

The proteins were fixed and stained with silver nitrate method or by Coomassie-staining [Laemmli, 1970]. For Western blotting, the proteins were transferred on PVDF Immobilon-P membranes (Millipore, MA) in 20 mM Tris–Gly buffer pH 8.3, 0.1% SDS, 20% methanol, using a BioRad blotter (400 mA for 90 min) (Richmond, CA). The saturation of non-specific binding sites was achieved by adding 5% no fatty milk in TBS buffer (10 mM Tris–HCl containing 9 g/l NaCl and 0.01% SDS) for 1 h at room temperature and washed five times with TBS. The membrane was then incubated with a rabbit polyclonal anti-Saporin serum (Sigma Aldrich, St. Louis, MA), overnight at +4°C and then washed five times in TBS. The membrane was then treated with a horse anti-rabbit IgG antibody conjugated with horseradish peroxidase (Vector, Burlingame, Canada) for 4 h at room temperature. After several washes with TBS, the membrane was revealed by the addition of 3,3-diaminobenzidine (0.6 mM), hydrogen peroxide (4.2 mM) and 4-chloro-1-naphtol (3.6 mM).

PURIFICATION OF hCASK-SAP AND $(hCASK)_2$-SAP CHIMERAS

The pellet of 1 l of culture of induced bacteria was resuspended in a 20 mM phosphate buffer pH 6.5 supplemented with proteases inhibitors (1 mg/ml pepstatin, 1 mg/ml leupeptin, 10 mg/ml aprotinin, and 15 mg/ml PMSF) and 1 mg/ml DNase. The suspension was sonicated and then centrifuged at 7,000*g* for 30 min at +4°C, to eliminate cells debris. The supernatant was acidified at pH 5 with diluted acetic acid, to precipitate contaminant proteins with acidic isoelectric point. This solution was centrifuged at 7,500*g* for 60 min at +4°C. This second supernatant was recovered, neutralized at pH 6.5 and dialyzed for 48–72 h, against 20 mM phosphate buffer pH 6.5 and filtered with 0.22 μm filters before chromatographic separation using an AKTA prime plus GE Healthcare FPLC-apparatus. For His-tagged proteins an HisTrap HP column, prepacked with Ni Sepharose High Performance (GE Healthcare, USA), was used. The column was equilibrated with 10 column volumes (CV) of binding buffer (20 mM phosphate buffer , 0,5 M NaCl, 50 mM imidazole, pH 7,4). After the sample was loaded, the column was washed with binding buffer. The elution was done with a continuous linear gradient from 0% to 70% elution buffer (20 mM phosphate Buffer, 0,5 M NaCl, 1 M imidazole, pH 7,4). The column equilibration and the elution were obtained using a constant flux of 1 ml/min. The eluted protein fractions were collected and successively analyzed by SDS-PAGE and Western blotting using anti His-Tag (Ab Cam, UK) or anti Saporin antibodies (Sigma Aldrich, USA). Once verified the presence and the purity of the protein, fractions containing it have been collected and dialyzed against PBS for the protein hCASK or D-MEM medium for the chimera hCASK-SAP or $(hCASK)_2$-SAP at 4°C for 12–24 h with changes of buffer every 6–8 h. The concentration of proteins were estimated by UV spectroscopy based on the theoretical extinction coefficient at 280 nm (http://web.expasy.org/protparam/). The chromatographic purification of Saporin from seeds was performed by ion-exchange chromatography on Resource S (Amersham Pharmacia) column in 20 mM phosphate buffer pH 6.5 and eluting with a linear NaCl salt gradient [Savino et al., 1998].

BIOCHEMICAL CHARACTERIZATION OF hCASK-SAP AND $(hCASK)_2$-SAP

Affinity measurements. The affinity of binding to target peptide sequences was assayed for both the fusion proteins by titration of the ligand binding site with N-terminal dansylated peptides. As a control we used the recombinant free PDZ domain of hCASK. A dansyl-peptide corresponding to the human sequence (RFPYAA) and one corresponding to the rat C-terminal sequence (QFPFVA) of CD98 were purchased from ProteoGenix SaS (Shiltigheim, France). The experiment was performed recording the fluorescence spectra of dansylated peptides (Exc. λ 340 nm, emission between 450 and 650 nm) mixing increasing amounts of free peptide with fixed protein (final concentrations 40 μM for the peptide, 1 μM for the protein). The binding of the peptide to the PDZ produced a quenching of the fluorescence signal revealed as a hyperbolic dependence of the maximum fluorescence (540 nm), while the free peptide or the unrecognized peptide gave a linear dependence. Data were fitted according to a single site ligand binding or when not possible to a linear regression equation using the software Grafit 5.0.1 (Erithacus Software Limited).

RIP activity on isolated ribosomes. Both hCASK-SAP and $(hCASK)_2$-SAP were assayed for their ability to release adenine from isolated ribosomes according to the following protocol.

Ribosomes from yeast (*S. cerevisiae*) were prepared according to standard procedures [Battaner and Vasquez, 1971]. Enzymatic reaction was obtained mixing proteins and ribosomes in a total volume of 200 μl at 37°C for 1 h. Negative control was performed mixing ribosomes with only PBS. Control with standards of adenine

was performed mixing ribosomes with progressive amounts of adenine (Sigma) in PBS. Reaction was stopped adding 1 vol. of cold ethanol and putting the samples 15 min at −80°C [Zamboni et al., 1989]. After 20′ of centrifugation, the supernatant was recovered and dried with a vacuum centrifuge. Pellet was suspended in MilliQ water (Millipore), loaded on 100,000 Da cut-off Microcon tubes (Millipore) and flow through was recovered.

Analysis were based on Yang method [Yang et al., 2007] using Waters Acquity UPLC I-Class system with PDA detector (Waters, MA), connected to a Waters Empower 3 software and equipped with Waters Acquity UPLC BEH C18 column (50 × 2.1mm i.d., particle size 1.7 mm). The column temperature was maintained at 25°C and the sample manager temperature was set at 10°C. Samples were separated using a gradient mobile phase consisting of 0.5 mM acetic acid (A) and acetonitrile (B). The gradient condition was: 0–5 min, 0–5% B; 5–6 min, 5–100% B; washing colum with 100% B for 1 min and isocratic reconditioning the column with 100% A for 3 min. The flow rate was set at 0.25 ml/min and the injection volume was 1 μl. The peaks were detected at 254 nm. The area corresponding to the peak revealed from free standard adenine (min. 1.7–1.9) was integrated using the instrument's software. A calibration curve was obtained running free adenine standards. hCASK-SAP and $(hCASK)_2$-SAP and saporin from seeds were tested. The amounts of adenine released by each toxin was extrapolated from the calibration curve. The data are reported as pmoles of adenine released/nmole of toxin present in the incubation mixture.

CELL LINES

The cell lines used in our experiments are GL15 (kindly provided by Prof.ssa Cristina Limatola, Department of Phisiology and Pharmacology, Faculty of Pharmacy and Medicine, University of Rome "La Sapienza") and U87 (ATCC Code: HBT-14). All the cells were cultured in Dulbecco's Modified Eagle's medium (D-MEM) supplemented with Penicillin (100 U.I./ml) Streptomycin (100 mg/ml), Glutamine (2,05 mM) and Fetal Bovine Serum (10%).

Both cell lines were tested for the expression of CD98 by immunofluorescence staining (data not shown) using a polyclonal anti CD98 (Santa Cruz, CA) as a primary antibody and a secondary TRITC-anti rabbit (SIGMA).

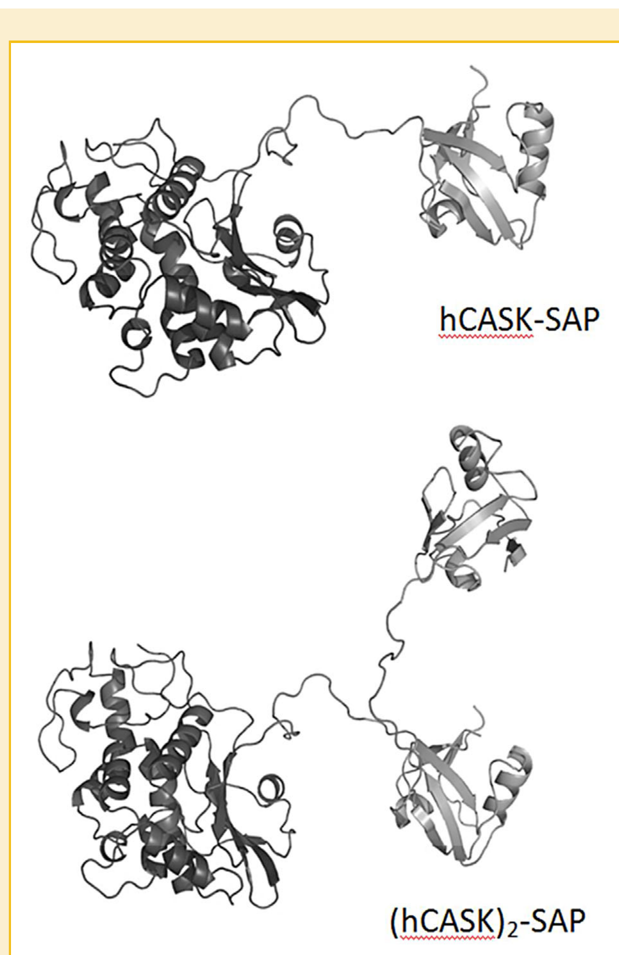

Fig. 1. Cartoon representation of the 3D models of hCASK-SAP (at the top) and $(hCASK)_2$-SAP (at the bottom) chimeric toxins is shown. The models were built with the program COOT [Emsley and Cowtan, 2004] starting from the available crystal structures of Saporin (PDB: 1QI7, in dark grey) and hCASK PDZ (PDB: 1KWA, in light grey). The crystal structures were linked by one and two $(G_4S)_3$ peptides in the case of hCASK-SAP and $(hCASK)_2$-SAP, respectively, and the resulting models have been subjected to geometry idealization. The figure was prepared using PyMol [The PyMOL Molecular Graphics System, Version 0.99rc6 Schrödinger, LLC].

CELL VIABILITY ASSAY WITH MTS TEST

2,000 cells/well, U87 and Gl15, were seeded in a 96 well plate in presence or absence of treatment (every treatment was performed in triplicate) and incubated for desired time (24, 48, 72 or 96 h). At expiration of incubation period cell viability was determined using the CellTiter 96AQueous One Solution Cell Proliferation assay. For this test, 20 μl of CellTiter 96AQueous One Solution Reagent containing a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) were added to each well containing 100 μl of medium and the cells were incubated for 1 h at 37°C. Living cells bio-reduced yellow MTS into a purple soluble formazan product with an absorbance peak at 492 nm, that was measured in a spectrophotometric microplate reader (Infinite F200 Tecan). The results are expressed as percentage of the absorbance of treated cells compared with control untreated cells.

In competition experiments on U87 cells, a fixed concentration of hCASK-SAP (10^{-10} M + SA1641) and $(hCASK)_2$-SAP (10^{-9}M) extrapolated from the IC_{50} values obtained in the cytotoxicity assay was applied to cells in the presence of 1, 10 or 100 equivalents of free anti CD98 (polyclonal, SantaCruz, CA). The results are expressed as percentage of the absorbance of treated cells compared with control untreated cells.

APOPTOSIS MEASUREMENTS

For apoptosis tests, 10,000 cells/well were seeded in a 6 well plate and incubated for 96 h with hCASK-SAP or $(hCASK)_2$-SAP at IC_{50} concentration calculated after cytotoxicity tests. At the expiration of the incubation the determination of cytoplasmic histone-associated DNA fragments was performed by using the Cell Death Detection ELISA Kit (Roche Diagnostics, CH) following the manufacturer's instructions. Briefly the assay is based on the quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. The absorbance peak at 405 nm was measured in a spectrophotometric microplate reader.

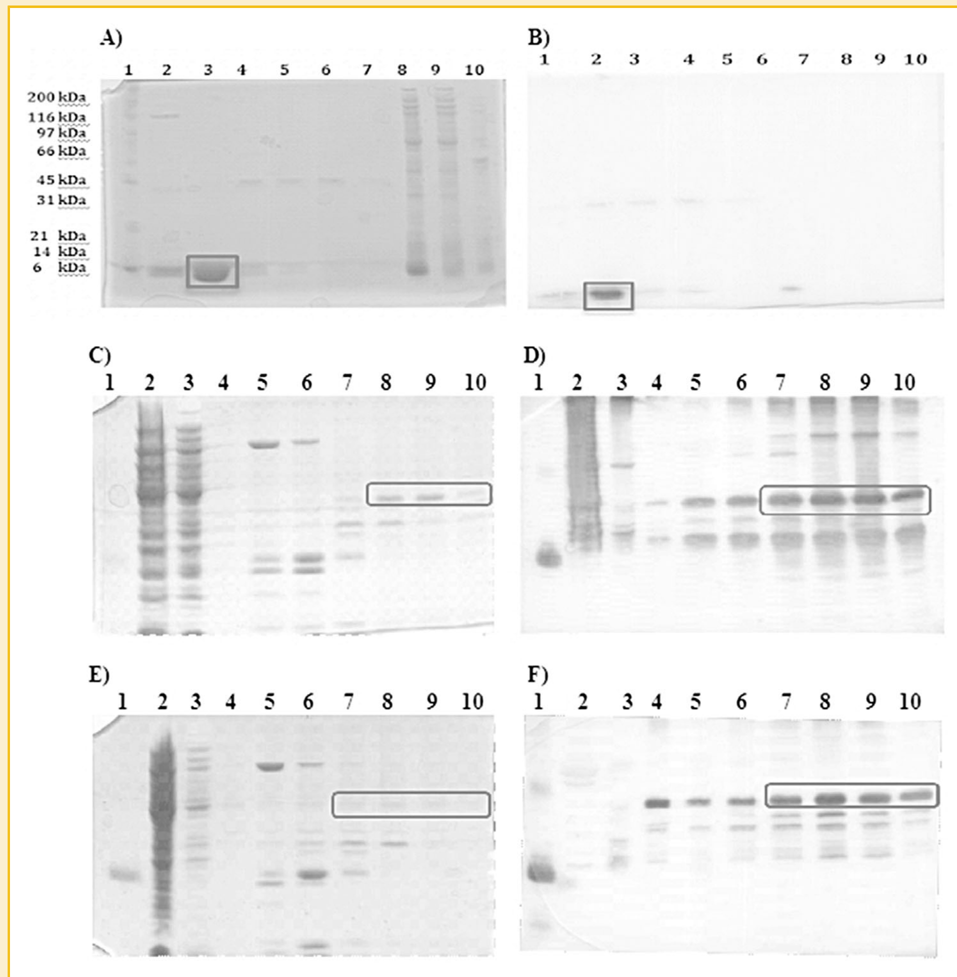

Fig. 2. SDS-PAGE Coomassie-stainded (A) and Western Blotting (B) of the hCASK eluted fractions obtained from IMAC. A) Lane1, MK; Lane2, Fr. 15; Lane3, Fr.23; Lane4, Fr.26; Lane5, Fr.33; Lane6, Fr.36, Lane7, Fr.42, Lane8, starting material; Lane9, Flow Through; Lane10, pellet. B) Lane1, Fr. 15; Lane2, Fr.23; Lane3, Fr.26; Lane4, Fr.33; Lane5, Fr.36, Lane6, Fr.42, Lane7, starting material; Lane7, Flow Through; Lane9, pellet. SDS-PAGE Coomassie-stainded (C) and Western Blotting (D) of the hCASK-SAP eluted fractions obtained from IMAC. Lane1, SAP-SOS [500ng]; Lane2, starting material; Lane3, Flow Through; Lane4, pellet; Lane5, Fr.18; Lane6, Fr.20, Lane7, Fr.26, Lane8, Fr. 32; Lane9, Fr.35; Lane10, Fr.40 SDS-PAGE Coomassie-stainded (E) and Western Blotting (F) of the $(hCASK)_2$-SAP eluted fractions obtained from IMAC. Lane1, SAP-SOS [500ng]; Lane2, starting material; Lane3, Flow Through; Lane4, pellet; Lane5, Fr.18; Lane6, Fr.22, Lane7, Fr.26, Lane8, Fr. 31; Lane9, Fr.35; Lane10, Fr.45

The results are expressed as percentage of absorbance, resulting from the activity of peroxidase conjugated anti-DNA antibody complexed with mono- and oligo-nucleosomes released into the cytoplasm of treated cells, compared with the control untreated cells. Statistical analysis of the data was obtained by two-tailed *P*-value calculation with the paired *t*-test using the software GraphPad (GraphPad Software, San Diego, CA, www.graphpad.com).

RESULTS

EXPRESSION, PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF CHIMERIC TOXINS

A 3D model of chimeric hCASK-SAP and $(hCASK)_2$-SAP chimeric toxins is reported in Figure 1. A single PDZ domain from hCASK was linked through a $(G_4S)_3$ peptide at the C-terminus of Saporin to obtain hCASK-SAP, while two consecutive PDZ domains (each separated by a $(G_4S)_3$ peptide) were linked to Saporin in the $(hCASK)_2$-SAP chimera.

The expression of hCASK single domain (9 kDa) from pET28, was performed in a *E.coli* BL21 (DE3) strain as a control of the production of recombinant proteins carrying this particular domain. Moreover the expression of hCASK-SAP (39 kDa) and $(hCASK)_2$-SAP (48 kDa) from pET11a in *E.coli* Rosetta Gami^{TM} B pLysS(DE3) or *E. coli* BL21 (DE3) respectively, was obtained.

After setting the culture/induction conditions (induction at 30°C for 4 h after administration of 1 mM IPTG) the screening of recombinants clones was performed and the best ones selected for scale-up protein expression. The electrophoretic and wb analysis of hCASK PDZ domain expression is reported in Figure 2. As shown (Fig. 2 panel A, lane3) hCASK eluted using a Imidazole gradient (0–300 mM) was particularly present in the fractions (black boxes)

corresponding to about 160 mM of Imidazole. In Figure 2B the Western blot analysis of the same fractions, using anti-HisTag, is reported.

The fractions obtained by IMAC for the hCASK-SAP or $(hCASK)_2$-SAP were analyzed by SDS–PAGE and Western blotting as well using as primary antibody a polyclonal anti-SAP antibody (Respectively Fig. 2C and D and Fig. 2E and F, black boxes for pertinent fractions). It is evident that their yields, in terms of expression, are greatly reduced in comparison with hCASK domain, as expected for the production of recombinant chimeras containing saporin, a toxin known to limit the expression of chimeras due to its intrinsic toxicity against host bacteria [Giansanti et al., 2010; Lombardi et al., 2010].

Despite the presence of immunoreactive bands related to aggregates or degradation products, the final purity of hCASK-SAP or $(hCASK)_2$-SAP was estimated to be over 80%. Both monovalent and bivalent chimeras are expressed with satisfactory levels (above 0.5 mg/L culture medium).

The biological activity of both hCASK-SAP and $(hCASK)_2$-SAP was tested as the ability to bind the target sequence of C-terminal CD98, using dansylated synthetic peptides. As reported in Table I both hCASK-SAP and $(hCASK)_2$-SAP retained the affinity towards the human peptide sequence of C-terminal CD98, if compared with the free PDZ from hCASK. They all showed affinity in the order of micromolar (Kd ranging from 6 to 15 μM). Notably none of the above tested proteins showed significant affinity towards a peptide derived from the rat CD98 C-terminal sequence (see supplementary Figures S1 and S2).

Furthermore we tested the ability of the same proteins in releasing adenine from isolated yeast ribsomes. As reported in Table II, the enzyme activity of hCASK-SAP and $(hCASK)_2$-SAP is comparable to that of seed saporin in the conditions used (see supplementary Figures S3–S5).

CELL VIABILITY INHIBITION

To evaluate the biological activity the two chimeric toxins (hCASK-SAP and $(hCASK)_2$-SAP) were tested on two glioblastoma cell lines (U87 and GL15). Figure 3 shows the MTS cell viability assay obtained for both chimeras or Saporin purified form seeds of *Saponaria officinalis* at reported concentrations (10^{-7}–10^{-13} M) and in the presence or absence of Saponin [2 μg/ml] as "coadiuvant" for endo/lysosomal compartment escape of the toxins. For each protein IC_{50} was calculated and reported in Table III.

hCASK-SAP induces cell death on both U87 and GL15 with a similar IC_{50} ($>10^{-8}$M) and the addition of the second PDZ domain on $(hCASK)_2$-SAP significantly increases the toxicity of this chimera on U87 cells but not in GL15 cells. These results may be partially attributed to the intrinsic lower activity of Saporin on GL15 cells, but also to different intracellular routes followed by the two toxins [Cimini et al., 2012]. In order to explore this possibility, we studied the effect of the addition of Saponin SA1641 from *Saponinum album* (an extract obtained from the roots of the plant *Gypsophila paniculata L.*). This substance has been already reported to greatly potentiate the toxicity of Saporin-based chimeras [Weng et al., 2009, 2012a,b] by acting on the intracellular transport of this toxin, and its possible role in the endosomal escape of Saporin has been suggested.

The addition of SA1641 induces a strong increase of Saporin and Saporin chimeras effects in U87 cells, as the IC_{50} values were lowered of at least three orders of magnitude. In GL15 cells we didn't observe any significant effect of SA1641, neither on Saporin nor on the two chimeras.

It is worth mentioning that hCASK PDZ domain alone did not induce any toxicity in both U87 and GL15 cells (data not shown) up to micromolar concentration, thus confirming that the observed toxicities were due to saporin component of the chimeras assayed in these experiments.

SPECIFICITY OF ACTION OF hCASK-SAP AND $(hCASK)_2$-SAP ON U87 CELLS

In order to assess the specificity of recognition of hCASK chimeras a competition experiment on U87 cells (sensitive to toxic action of both chimeras) was performed, due to their higher sensitivity to these toxins. As shown in Figure 4 the addition of an excess anti-CD98 competes with the toxicity of both hCASK-SAP and $(hCASK)_2$-SAP. In the case of hCASK-SAP the experiment was performed in the presence of SA1641, a condition necessary to get a measurable IC_{50} for this toxin on U87 cells. Higher concentrations of anti-CD98 induced higher cytotoxicity (data not shown) hampering the study on the inhibitory effects at higher Ab/toxin ratios.

CELL VIABILITY: APOPTOSIS

In order to establish the pro-apoptotic potential of the two chimeric-toxins (hCASK-SAP and $(hCASK)_2$-SAP the levels of cytoplasmic

TABLE I. Kd Values Calculated From Fitting Curves (See Figures S1-S2) According to a Single Site Ligand Binding Equation

Type of protein	Kd (μM)
hCASK-PDZ	16,2 + 5
hCASK-SAP	7,3 ± 2
$(hCASK)_2$-SAP	9,6 ± 4

TABLE II. Adenine Release From Yeast Ribosomes Calculated From Integration of UPLC Chromatographic Analysis (see Figures S3–S5)

Type of protein	Picomoles of adenine released/nanomole of protein
SAP-SO6	220.7
hCASK-SAP	215.3
$(hCASK)_2$-SAP	224.3

TABLE III. IC_{50} Calculated for hCASK-SAP, hCASK)$_2$-SAP nd SAP-SO6 Against U87 and GL15 Cell Lines in the Presence or Absence of SPN [2 μg/ml]. IC_{50} Values Have Been Extrapolated From Fitting Curves

	IC_{50} (M)		
	hCASK-SAP	$(hCASK)_2$-SAP	SAP-SO6
U87 − SPN	$>>10^{-8}$	5×10^{-9}	5×10^{-7}
U87 + SPN	5×10^{-10}	$<<10^{-12}$	$<10^{-11}$
GL15 − SPN	10^{-7}	5×10^{-6}	$>10^{-6}$
GL15 + SPN	10^{-7}	10^{-6}	$>10^{-6}$

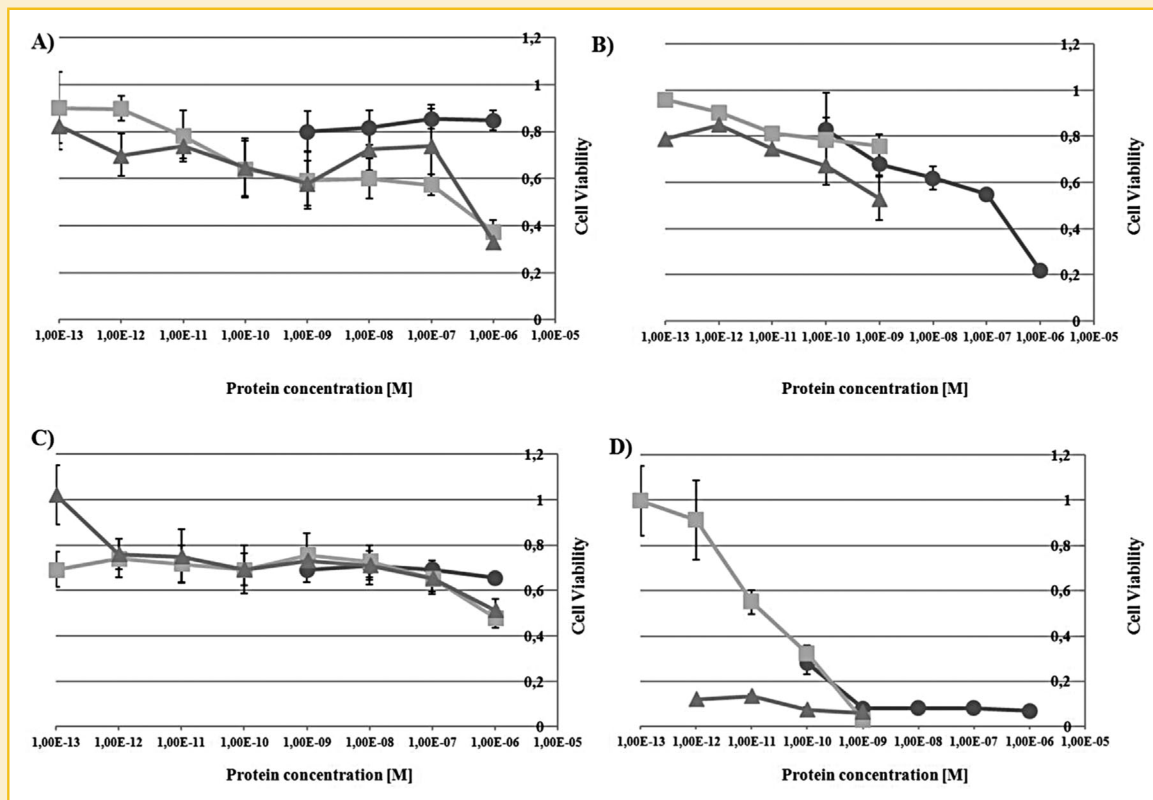

Fig. 3. MTS Cell viability assay on GL15 (A and C) or U87 (B and D) cells treated with with Saporin purified from seeds of *Saponaria officinalis* (●) SAP-SO6 or with the two chimera toxins (■) hCASKSAP and (▲) $(hCASK)_2SAP$) in absence (A and B) or presence (C and D) of Saponin SA1641 [2 μg/ml]. In both cases the proliferation was calculated using control cells as 100% of viability. The corresponding IC_{50} was calculated from fitting curves obtained with the software Graphit (see methods) and reported in Table III.

nucleosomes after 96 h of treatment both on U87 and GL15 cell lines) were evaluated. In Figure 5 it is evident that the $(hCASK)_2$-SAP toxin is more effective than hCASK-SAP towards U87 cell line. It is worth noting that both chimeric toxins exhibit significant ability to induce apoptosis in comparison with free saporin at doses that are one or two orders of magnitude lower in U87 cells, while the two toxins have low and similar pro-apoptotic effects on GL15 cells in comparison to the free toxin.

DISCUSSION

In this work we engineered a PDZ protein domain as possible tool for cellular targeting of the ribosome inactivating protein Saporin, with the aim of proposing proof of concept for designing new systems to target cancer cells. We wished to exploit the ability of PDZ domains to recognize and bind short peptide sequences usually found at the C-terminus of cognate proteins in cellular protein-protein interaction phenomena, such as organization of transport, localization, sorting and spatial arrangement of proteins. Our studies have been focused on the PDZ domain from hCASK (Human calcium/calmodulin-dependent serine protein kinase) that has been demonstrated to bind extracellular CD98 in epithelial cells [Yan et al., 2007].

This antigen has been recently studied and recognized as a marker for several human tumors [Cantor and Ginsberg, 2012] and particularly considered a negative prognostic marker for human glioblastoma [Nawashiro et al., 2002; Takeuchi et al., 2008].

We linked PDZ domain of hCASK to Saporin, a well-studied toxin that has been used for the production of chimeric immunotoxins [Flavell et al., 1991; Flavell 1998; Fabbrini et al., 2003; Lombardi et al., 2010] and so far is one of the most useful tools in the neurobiology research [Lappi and Wiley, 2004]. We produced fusions of one or two hCASK-PDZ domains with Saporin and tested it on two human glioblastoma model cell lines (GL15 and U87). Both hCASK-SAP chimeras (carrying one or two PDZ domains, see Figure 1) maintain the biological properties of the original macromolecular components (hCASK-PDZ and saporin) and induce cell cytotoxicity and cell death by apoptotic mechanisms in a dose-dependent and/or time dependent manner, with increasing activity as a function of the number of PDZ domains. We could estimate toxicity towards glioblastoma U87 with an IC_{50} value for hCASK-SAP greater than that of free Saporin (around 10^{-7} M), furthermore a significant increase in toxicity was observed upon addition of a second hCASK domain to Saporin, $(hCASK)_2$-SAP, driving IC_{50} value down to 10^{-9}M, allowing this latter fusion to be two orders of magnitude more effective than the free Saporin toxin.

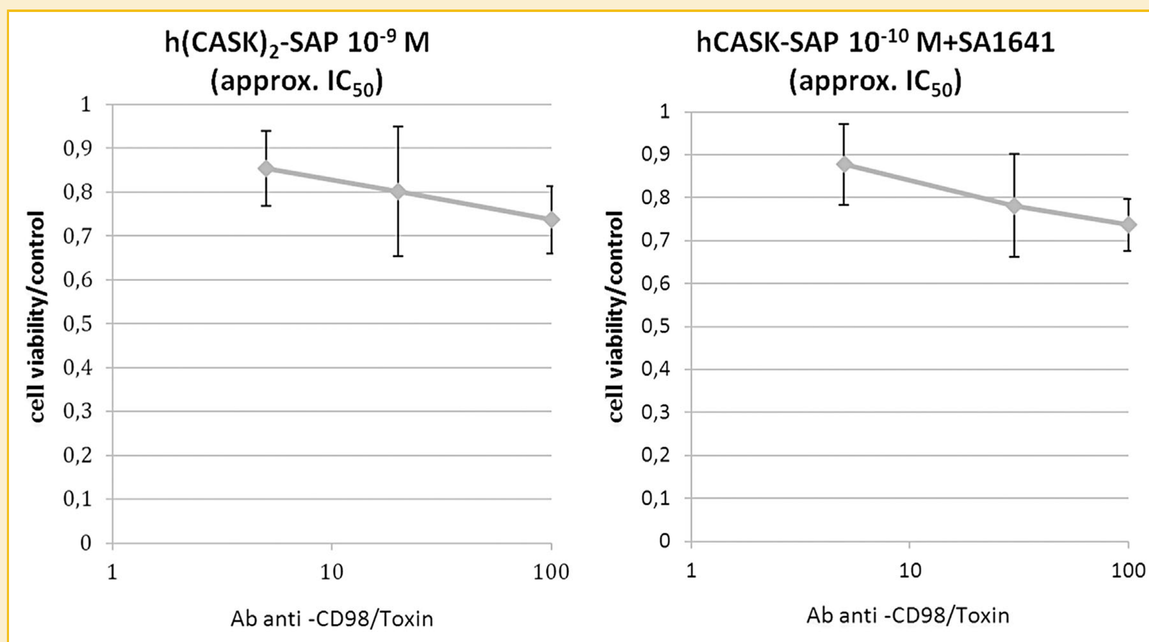

Fig. 4. Cell viability on U87 cells treated with $h(CASK)_2$-SAP or hCASK-SAP +SA1641 at their respective IC_{50} concentrations (in the presence of 2 μg/ml SA1641 for hCASK-SAP) in the presence of anti CD98 polyclonal antibody.

Surprisingly, even though GL15 cells express CD98 on the cell surface, we didn't observe significant different toxicities for both hCASK-Saporin fusions if compared to free Saporin, being their IC_{50} around 10^{-7}M. The therpenic compound Saponin SA1641, known to increase the toxicity of Saporin-based chimeras in many cell lines [Weng et al., 2012a, b] drastically increased the toxicity of Saporin and $h(CASK)_2$-SAP in U87 cells (IC_{50} $<10^{-11}$M and $<<10^{-12}$M respectively), and allowed hCASK-SAP to have an IC_{50} of 10^{-10}M. On the other hand, SA1641 didn't produce any increase of toxicity in GL15 cells for any of the toxins studied.

These results in U87 cells may be indicative of intracellular delivery passing through the endosomal transport, where Saponin SA1641 can exert its potent release of Saporin, but also indicate that intracellular delivery mediated by a single hCASK domain may not be efficient in releasing Saporin moiety inside the cell cytoplasm. In fact the addition of a second PDZ domain, greatly increases the toxicity of Saporin, in U87 cells.

In GL15 cells, the cellular response seems to be mediated by Saporin internalization mechanisms rather than hCASK delivery, since we couldn't observe differences between the toxins even in the presence of SA1641, suggesting in these cells an intracellular delivery that differs from that obtained in U87 cells. We previously did observe a differential sensitivity of GL15 cells to Saporin and its transferrin-conjugates [Cimini et al., 2012], partially due to the presence in this cell line of a mutated p53, a phenomenon that has been interpreted as the need of a functional DNA damage sensor system to allow Saporin toxicity to be exerted [Cimini et al., 2012].

The observation that a second hCASK domain may increase the toxic potential of Saporin, is confirmed by the induction of apoptosis, since in U87 cells (differentially sensitive to Saporin and hCASK fusions) we observed a clear increase of apoptotic cell death as a function of the number of hCASK domains both at 10^{-8}M and 10^{-7}M concentrations. Again the pro-apoptotic effect on GL15 cells of the two hCASK-containing chimeras is reduced and similar.

We tested the specificity of the toxic effect on U87 cells by competing the toxin activity with an excess anti CD98 antibody, thus confirming that the intracellular delivery of hCASK-SAP and $h(CASK)_2$-SAP is dependent on the binding of the PDZ domain of hCASK to the membrane receptor. This experiment is significant to establish that this protein-protein interactions have enough selectivity to be proposed for the delivery of toxic molecules to tissues over-expressing CD98.

Thus, the results obtained suggest that increasing the number of PDZ domains (hCASK) may be a strategy to obtain efficient delivery of toxic cargos in cancer cells, exploiting their selectivity to CD98. Further combination of PDZ domains, with other selectivity, could be in principle used to engineer multivalent targeting systems, allowing the creation of chimeric molecules able to recognize different targets on the surface of cancer cells. This multi-target approach may be useful to efficiently kill cancer cells in a heterogeneous population, where the lower expression of a single antigen may be limiting to have good therapeutic effects upon treatment in vivo with targeted toxins [Flavell et al., 1997].

Furthermore the possibility of conjugation of our chimeras to nanoparticles such as dendrimers, carbon nanotubes, liposomes, and micelles may be a possible future application to improve the drug delivery and bioavailability in the nervous system district.

Since to our knowledge this is the first description of the use of a PDZ domain in drug targeting, this strategy could open new perspectives in the creation of innovative drugs in the next future.

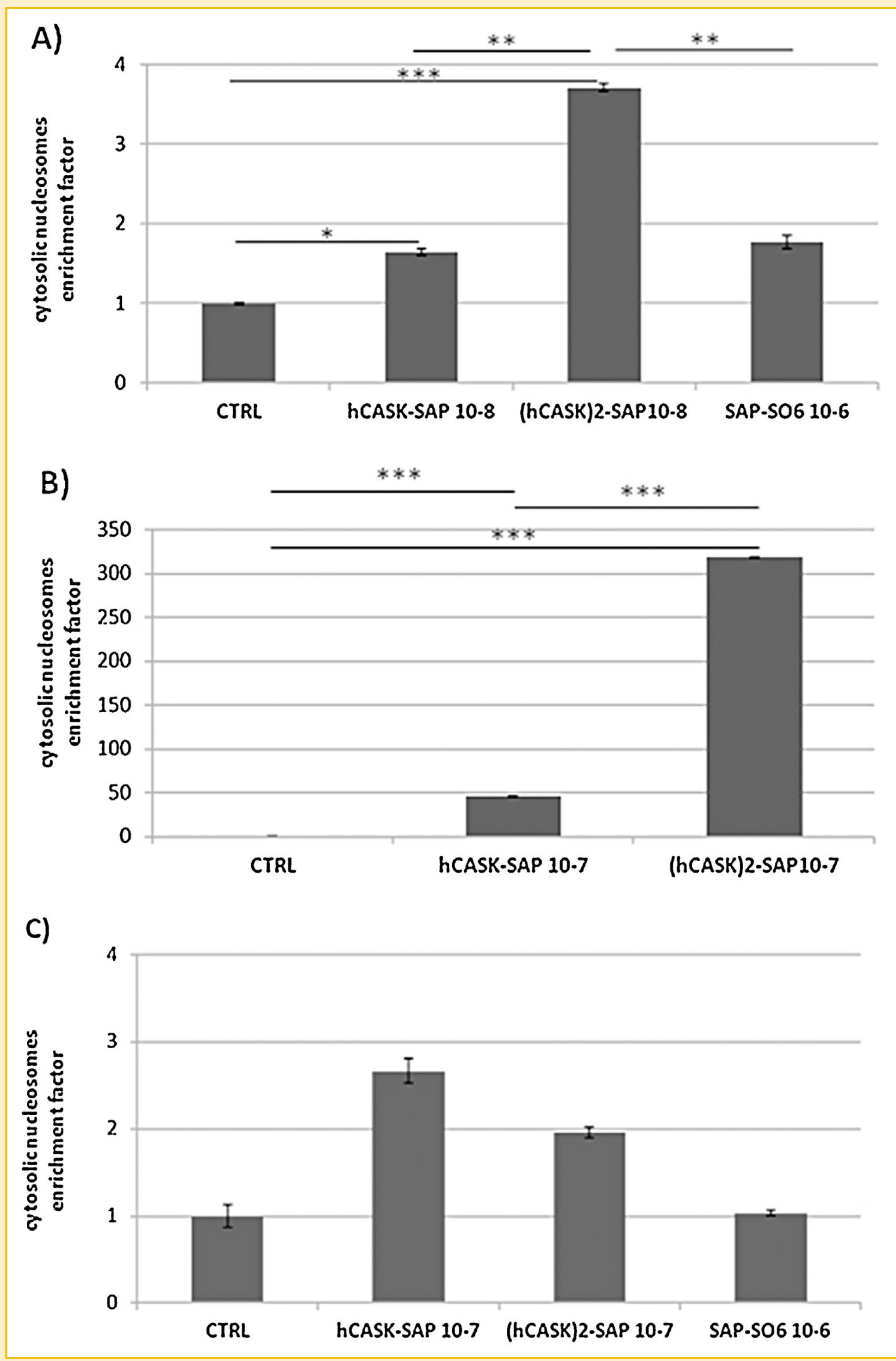

Fig. 5. Apoptosis measured on U87 and GL15 cells treated for 96 h with the two chimeric toxins hCASK–SAP, $(hCASK)_2$–SAP at concentration corresponding to IC_{50} reported in Table I (Panel A for U87 cell line, Panel C for GL15 cell line). Panel B shows the net apoptosis augmentation for U87 cell lines, depending on the increase of the concentration of the constructs (10^{-7} M) and/or form increasing number of hCASK domains . In the figures are reported in the ordinate the enrichment of cytosolic nucleosomes compared to the control cells untreated. (* $P < 0.1$; ** $P < 0.01$; *** $P < 0.0001$).

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REFERENCES

Anselmo AC, Mitragotri S. 2014. An overview of clinical and commercial impact of drug delivery systems. J Control Release 190:15–28.

Bachran D, Schneider S, Bachran C, Weng A, Melzig MF, Fuchs H. 2011. The endocytic uptake pathways of targeted toxins are influenced by synergistically acting Gypsophila saponins. Mol Pharm 8(6):2262–2272.

Battaner E, Vasquez D. 1971. Preparation of active 60S and 40S subunits from yeast ribosomes. Methods Enzymol 20:446–449.

Cantor JM, Ginsberg MH. 2012. CD98 at the crossroads of adaptive immunity and cancer. J Cell Sci 125:1373–1382.

Cho KO, Hunt CA, Kennedy MB. 1992. The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron 9:929–942.

Cimini A, Mei S, Benedetti E, Laurenti G, Koutris I, Cinque B, Cifone MG, Galzio R, Pitari G, Di Leandro L, Giansanti F, Lombardi A, Fabbrini MS, Ippoliti R. 2012. Distinct cellular responses induced by saporin and a transferrin-saporin conjugate in two different human glioblastoma cell lines. J Cell Physiol 227(3):939–951.

Dave MH, Schulz N, Zecevic M, Wagner CA. 2004. Expression of heteromeric amino acid transporters along the murine intestine. J Physiol 558:597–610.

Fabbrini MS, Flavell DJ, Ippoliti R. 2003. Plant protein toxins: Structure, function and biotechnological applications. In: Ascenzi P, Polticelli F, Visca P, editor., Bacterial Plant and Animal Toxins. Trivandrum, India: Research Signpost. pp 69–99.

Emsley P, Cowtan K. 2004. Coot: Model-building tools for molecular graphics. Acta Crystallographica D60:2126–2132.

Fenczik CA, Zent R, Dellos M, Calderwood DA, Satriano J, Kelly C, Ginsberg MH. 2001. Distinct domains of CD98hc regulate integrins and amino acid transport. J Biol Chem 276:8746–8752.

Flavell DJ. 1998. Saporin immunotoxins. Curr Top Microbiol Immunol 234:57–61.

Flavell DJ, Cooper S, Morland B, Flavell SU. 1991. Characteristics and performance of a bispecific F (ab′gamma) 2 antibody for delivering saporin to a CD7+ human acute T-cell leukaemia cell line. Br J Cancer 64(2):274–280.

Flavell DJ, Noss A, Pulford KA, Ling N, Flavell SU. 1997. Systemic therapy with 3BIT, a triple combination cocktail of anti-CD19, -CD22, and -CD38-saporin immunotoxins, is curative of human B-cell lymphoma in severe combined immunodeficient mice. Cancer Res 1; 57(21):4824–4829.

Gianni S, Engström Å, Larsson M, Calosci N, Malatesta F, Eklund L, Ngang CC, Travaglini-Allocatelli C, Jemth P. 2005. The kinetics of PDZ domain-ligand interactions and implications for the binding mechanism. J Biol Chem 280:34805–34812.

Gianni S, Walma T, Arcovito A, Calosci N, Bellelli A, Engström Å, Travaglini-Allocatelli C, Brunori M, Jemth P, Vuister GW. 2006. Demonstration of long-range interactions in a PDZ domain by NMR, kinetics and protein engineering. Structure 14:1801–1809.

Giansanti F, Di Leandro L, Koutris I, Pitari G, Fabbrini MS, Lombardi A, Flavell DJ, Flavell SU, Gianni S, Ippoliti R. 2010. Engineering a switchable toxin: the potential use of PDZ domains in the expression, targeting and activation of modified saporin variants. Protein Eng Des Sel 23(2):61–68.

Harris BZ, Lim WA. 2001. Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci 114:3219–3231.

Ippoliti R, Lendaro E, Bellelli A, Fiani ML, Benedetti PA, Evangelista V, Brunori M. 1996. A saporin-insulin conjugate: synthesis and biochemical characterization. Nat Toxins 4(4):156–162.

Jemth P, Gianni S. 2007. PDZ Domains: Folding and Binding. Biochemistry 46(30):8701–8708.

Kanai Y, Segawa H, Miyamoto K, Uchino H, Takeda E, Endou H. 1998. Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). J Biol Chem 273:23629–23632.

Kobayashi K, Ohnishi A, Promsuk J, Shimizu S, Kanai Y, Shiokawa Y, Nagane M. 2008. Enhanced tumor growth elicited by L-type amino acid transporter 1 in human malignant glioma cells. Neurosurgery 62(2):493–503.

Kurakin A, Swistowski A, Wu SC, Bredesen DE. 2007. The PDZ domain as a complex adaptive system. PLoS One 26:2(9):e953.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 5 227(5259):680–685.

Lappi DA, Wiley RG. 2004. Immunotoxins and neuropeptide-toxin conjugates experimental applications. Mini Rev Med Chem 4(5):585–595.

Lombardi A, Bursomanno S, Lopardo T, Traini R, Colombatti M, Ippoliti R, Flavell DJ, Flavell SU, Ceriotti A, Fabbrini MS. 2010. Pichia pastoris as a host for secretion of toxic saporin chimeras. FASEB J 24(1):253–265.

Merlin D, Sitaraman S, Liu X, Eastburn K, Sun J, Kucharzik T, Lewis B, Madara JL. 2001. CD98-mediated links between amino acid transport and beta 1 integrin distribution in polarized columnar epithelia. J Biol Chem 276: 39282–39289.

Nawashiro H, Otani N, Shinomiya N, Fukui S, Nomura N, Yano A, Shima K, Matsuo H, Kanai Y. 2002. The role of CD98 in Astrocytic Neoplasms. Human Cell 15(1):25–31.

Nguyen HT, Dalmasso G, Yan Y, Laroui H, Charania M, Ingersoll S, Ayyadurai S, Zhang Y, Sitaraman S, Merlin D. 2012. Intestinal epithelial cell-specific CD98 expression regulates tumorigenesis in Apc(Min/ +) mice. Lab Invest 92(8):1203–1212.

Okubo S, Zhen HN, Kawai N, Nishiyama Y, Haba R, Tamiya T. 2010. Correlation of L-methyl-11C-methionine (MET) uptake with L-type amino acid transporter 1 in human gliomas J Neurooncol 99(2):217–225.

Rossier G, Meir C, Bauch C, Summa V, Sordat B, Verrey F, Kuhn CJ. 1999. LAT2, a New Basolateral 4F2hc/CD98-associated Amino Acid Transporter of Kidney and Intestine. J Biol Chem 274:39948–39954.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, Second Edition. (Plainview, New York: Cold Spring Harbor Laboratory Press).

Sanford JL, Mays TA, Rafael-Fortney JA. 2004. CASK and Dlg form a PDZ protein complex at the mammalian neuromuscular junction. Muscle Nerve 20:164–171.

Savino C, Federici L, Brancaccio A, Ippoliti R, Lendaro E, Tsernoglou D. 1998. Crystallization and preliminary X-ray study of saporin, a ribosome-inactivating protein from Saponaria officinalis. Acta Crystallogr D Biol Crystallogr 1(54):636–638.

Takeuchi H, Kubota T, Kitai R, Nakagawa T, Hashimoto N. 2008. CD98 immunoreactivity in multinucleated giant cells of glioblastomas: An immunohistochemical double labeling study. Neuropathology 28:127–131.

Verrey F, ClossI EI, Wagner CA, Palacin M, Endou H, Kanai Y. 2004. CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch 447(5):532–542.

Weng A, Görick C, Melzig MF. 2009. Enhancement of toxicity of saporin-based toxins by Gypsophila saponins–kinetic of the saponin. Exp Biol Med (Maywood) 234(8):961–966.

Weng A, Thakur M, Beceren-Braun F, Bachran D, Bachran C, Riese SB, Jenett-Siems K, Gilabert-Oriol R, Melzig MF, Fuchs H. 2012b. The toxin component of targeted anti-tumor toxins determines their efficacy increase by saponins. Mol Oncol 6(3):323–332.

Weng A, Thakur M, von Mallinckrodt B, Beceren-Braun F, Gilabert-Oriol R, Wiesner B, Eichhorst J, Böttger S, Melzig MF, Fuchs H. 2012a. Saponins modulate the intracellular trafficking of protein toxins. J Control Release 28 164(1):74–86.

Yan Y, Vasudevan S, Nguyen H, Bork U, Sitaraman S, Merlin D. 2007. Extracellular interaction between hCD98 and the PDZ class II domain of hCASK in intestinal epithelia. J Memb Biol 215:15–26.

Yan Y, Vasudevan S, Nguyen H, Merlin D. 2008. Intestinal. Epithelial C D98: An Oligomeric and Multifunctional protein. Biochim Biophys Acta 1780-(10):1087–1092.

Yanagida O, Kanai Y, Chairoungdu A, Kim DK, Segawa H, Nii T, Cha SH, Matsuo H, Fukushima J, Fukasawa Y, Tani Y, Taketani Y, Uchino H, Kim JY, Inatomi J, Okayasu I, Miyamoto K, Takeda E, Goya T, Endou H. 2001. Human 1-type amino acid transporter 1 (LAT1): Characterization of function and expression in tumor cell lines. Biochim Biophys Acta 1514:291–302.

Yang FQ, Guan J, Li SP. 2007. Fast simultaneous determination of 14 nucleosides and nucleobases in cultured Cordyceps using ultra-performance liquid chromatography. Talanta 73(2):269–273.

Zamboni M, Brigotti M, Rambelli F, Montanaro L, Sperti S. 1989. High-pressure-liquid-chromatographic and fluorimetric methods for the determination of adenine released from ribosomes by ricin and gelonin. Biochem J 259(3):639–643.

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