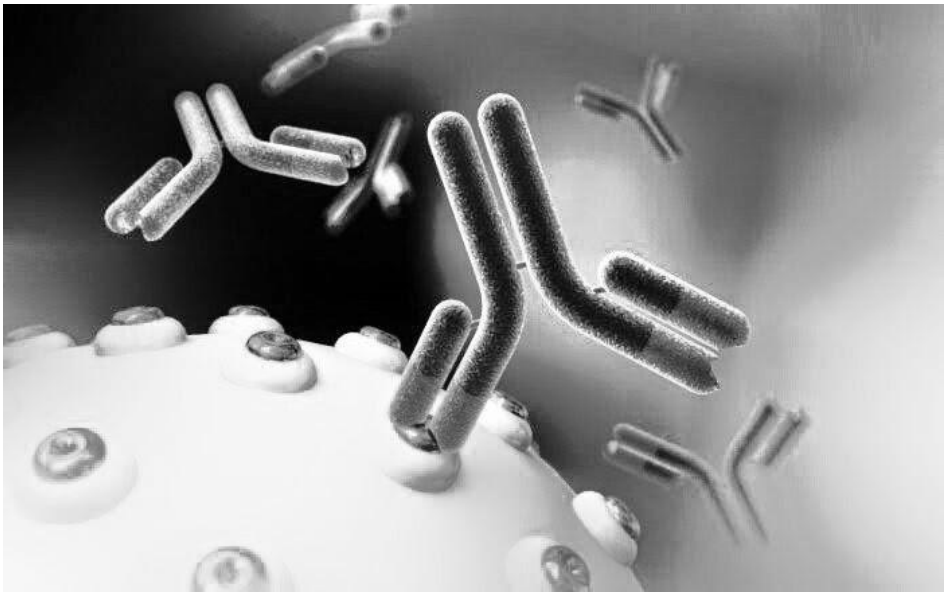
# UNIVERSITA' DI NAPOLI FEDERICO II

**DOTTORATO DI RICERCA IN BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE**
**XXVII CICLO**

**Paciello Rolando**

## *Human antitumor and antiviral Immunoagents*



**Academic Year 2013/2014**

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## *Human antitumor and antiviral Immunoagents*


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## Riassunto

L'immunoterapia rappresenta una valida strategia per le terapie antitumorali e antivirali. La tecnologia del *phage display* ha consentito la produzione di immunoagenti completamente umani specifici per antigeni associati a tumore (TAA) o per recettori cellulari di superficie essenziali per l'infezione virale.

Mediante l'uso di tale tecnologia abbiamo isolato frammenti anticorpali (scFvs) umani specifici per i loops extracellulari della Claudina-1 (CLDN-1), proteina transmembrana che forma tight junctions ed è essenziale per l'ingresso e per l'infezione del virus dell'epatite C (HCV). Gli scFvs selezionati sono stati convertiti in anticorpi umani con isotipo IgG4 e caratterizzati. Essi mostrano una selettiva ed elevata affinità di legame per cellule esprimenti la proteina CLDN-1, riconoscono diversi epitopi della proteina ed inibiscono efficientemente con modalità dose-dipendente l'infezione da HCV in colture cellulari di epatociti umani.

Un bersaglio utile per l'immunoterapia del cancro è rappresentato da ErbB2, un recettore ad attività tirosina-chinasica iper-espresso su diversi tipi di cellule tumorali, tra cui quelle della ghiandola mammaria, mentre è espresso a bassi livelli su cellule normali e solo su alcuni tipi di cellule epiteliali.

Un nuovo frammento anticorpale umano (scFv), chiamato Erbicin, specifico per il recettore ErbB2 era stato ottenuto nel nostro laboratorio mediante tecnologia del *phage display*.

Erbicin è stato fuso ad una variante della ribonucleasi pancreatica umana (HP-RNasi) resistente all'inibitore delle RNasi, in modo da ottenere una nuova immunoRNasi anti-ErbB2, chiamata Erb-HPDDADD-RNasi. In particolare, la nuova immunoRNasi è in grado di legare con elevata affinità un pannello di cellule tumorali gastriche esprimenti diversi livelli di ErbB2 e di inibire la loro crescita più efficientemente rispetto all'immunoRNasi parentale, sensibile all'inibitore. Inoltre, Erb-HP-DDADD-RNasi ha mostrato attività citotossica anche per cellule tumorali resistenti a Herceptin, sia *in vitro* che *in vivo* in modelli sperimentali di topo.

Simili risultati sono stati ottenuti dalla fusione di Erbicin con il frammento Fc di una IgG1 umana in modo da ottenere un costrutto, chiamato Erb-hcAb (anticorpo compatto anti-ErbB2) per le sue dimensioni ridotte (100 kDa), rispetto a quelle di una IgG umana (155 kDa).

Nel presente lavoro abbiamo studiato gli effetti anti-tumorali su cellule di carcinoma gastrico di Erb-hcAb, che è in grado di riconoscere un epitopo di ErbB2 diverso rispetto a quello di trastuzumab (Herceptin) e pertuzumab (Omnitarg), gli unici anticorpi anti-ErbB2 attualmente in uso clinico per la terapia del carcinoma mammario. I risultati dimostrano che la crescita di cellule tumorali gastriche è efficientemente inibita sia in vitro che in vivo da Erb-hcAb, il quale mostra un effetto antitumorale sulla linea cellulare NCI-N87 anche più potente di quella osservata per Herceptin.

In conclusione, tali nuovi immunoagenti umani possono rappresentare un valido strumento per la terapia antitumorale e antivirale in associazione o in alternativa alle attuali terapie convenzionali.

## Summary

Immunotherapy is a precious strategy to fight cancer and viral infections. Phage display technology allows for the production of fully human immunoagents specific for tumor associated antigens (TAA) or for cell surface receptors involved in virus infection.

By using this technology we isolated fully human antibody fragments (scFvs) specific for the extracellular loops of Claudin-1 (CLDN-1), a tight junction protein essential for hepatitis C virus (HCV) cell entry and following infection. The selected scFvs have been converted in human IgG4 antibodies and characterized. They show high selective binding affinities for CLDN-1-positive cells, recognize distinct epitopes of the protein, and specifically inhibit HCV infection in a dose-dependent manner in cell cultures of human hepatocytes.

An attractive TAA for cancer immunotherapy is ErbB2, a tyrosine kinase receptor overexpressed on many different types of tumor cells, such as breast and gastric cancer, whereas it is expressed at low levels on normal cells and only on certain epithelial cell types.

A human antibody fragment (scFv), named Erbicin, specific for ErbB2 receptor has been isolated in our laboratory by using phage display technology.

Erbicin was fused with a human pancreatic RNase (HP-RNase) variant resistant to the cytosolic inhibitor, to obtain a novel anti-ErbB2 immunoRNase (IR), which was called Erb–HPDDADD-RNase. The novel IR binds with high affinity to a panel of ErbB2-positive gastric tumor cells and inhibits their growth more efficiently than the parental immunoRNase, which is not resistant to the inhibitor. Moreover, Erb–HP-DDADD-RNase is endowed with an antitumor activity for Herceptin-resistant cancer cells both *in vitro* and *in vivo*.

Similar results were obtained fusing Erbicin with the Fc region of a human IgG1 to obtain a construct, which was called *Erb-hcAb* for its compact size (100 kDa) if compared with the full size (155 kDa) of a natural IgG.

Here we report on the antitumor effects on gastric cancer cells of Erb-hcAb which targets a different epitope of ErbB2 with respect to trastuzumab (Herceptin) and pertuzumab (Omnitarg), the only anti-ErbB2 antibodies currently in clinical use for breast cancer therapy. The results demonstrate that the growth of gastric cancer cells is efficiently inhibited *in vitro* and *in vivo* by Erb-hcAb, which shows antitumor effects on the NCI-N87 cell line more potent than those observed for Herceptin.

In conclusion, these human immunoagents may represent valuable tools for both antitumor and antiviral therapies in association or in alternative to conventional therapies currently used.

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# INTRODUCTION

## *1. Human antiviral immunoagents*

### *1.1. Hepatitis C Virus*

Hepatitis C virus (HCV) infection is one of the most common global health diseases. HCV is a member of the Flaviviridae family and Hepacivirus genus. The virion contains a single-stranded positive RNA with a length of about 10.000 nucleotides, consisting of a single ORF which is encapsulated by an icosahedral capsid and an external lipid envelope. HCV is a heterogeneous virus, classified into 7 genotypes and more than 50 subtypes with a genome variability of 30-35% among genotypes, and of 20-25% among subtypes (1).

This genetic variability in the virus populations provides a survival advantage to the virus by creation of multiple variant genomes and a high rate of variant generations, that allow for a rapid selection of mutants in new environmental conditions.

In the virus genome there are two UTRs located at the 5' and 3' termini called cis-active RNA elements which are essential for protein translation. A single precursor polyprotein is approximately composed by 3010 aminoacids, cleaved into 10 different proteins. Structural Core proteins, E1, E2 and p7 are released from the polyprotein after cleavage by cellular proteases; the non-structural proteins such as NS2, NS3 and NS4A are produced by virus-encoded proteases (2, 3).

The E1 and E2 envelop proteins are highly glycosylated and play an important role in cell entry, which is a slow and complex process consisting of many steps. The initial attachment of the viral protein E2 is to glycosaminoglycans (GAGs) and Low Density Lipoprotein receptor (LDL-R), followed by interaction with a complex formed by SR-BI and CD81. Virus associated to CD81 is subsequently transferred to tight junctions due the association of CD81 with CLDN-1. HCV enters the cell by clathrin-mediated endocytosis and, upon acidification, the fusion of the virus envelope leads to the release of the viral genome into the cytoplasm (4) (Figure 1).

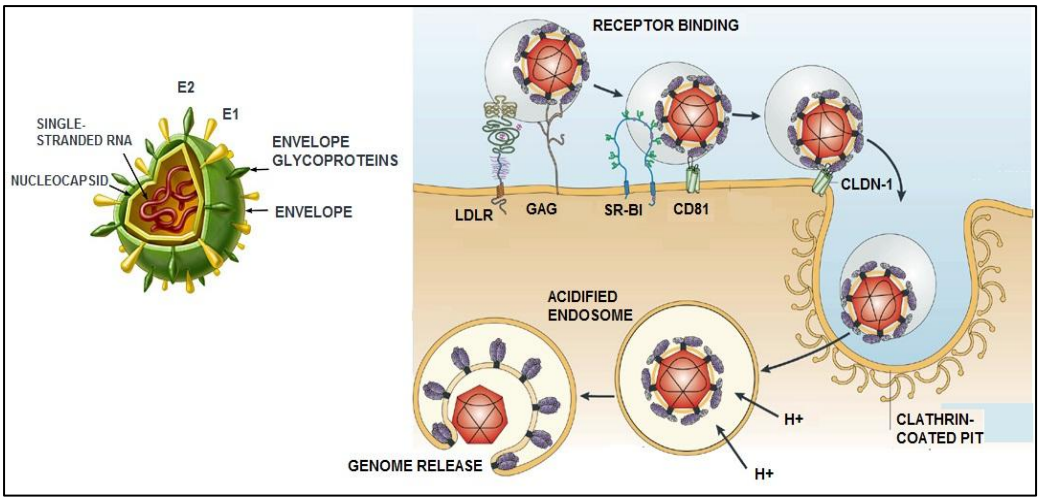



**Fig. 1 - HCV structure and its cell-entry mechanism**

Claudins are the most important structural and functional components of tight junction integral membrane proteins expressed in all epithelial tissues, with at least 24 members in mammals. They are crucial for the paracellular flux of ions and small molecules. Claudin-1 (CLDN-1) is expressed predominantly in the liver, where it localizes both at tight junctional (apical-canalicular) and extrajunctional (basolateral-sinusoidal) sites of hepatocytes, but predominantly at the apical site.

The structure of Claudin-1 consists of four transmembrane helices, with a very short intracellular N-terminal sequence (2–6 residues), two extracellular loops, and an intracellular C terminal tail (5) (Figure 2). The first loop influences paracellular charge selectivity (6), the second loop is the receptor for a bacterial toxin (7), and the C terminus binds to cytoplasmic proteins through a PDZ motif (8).

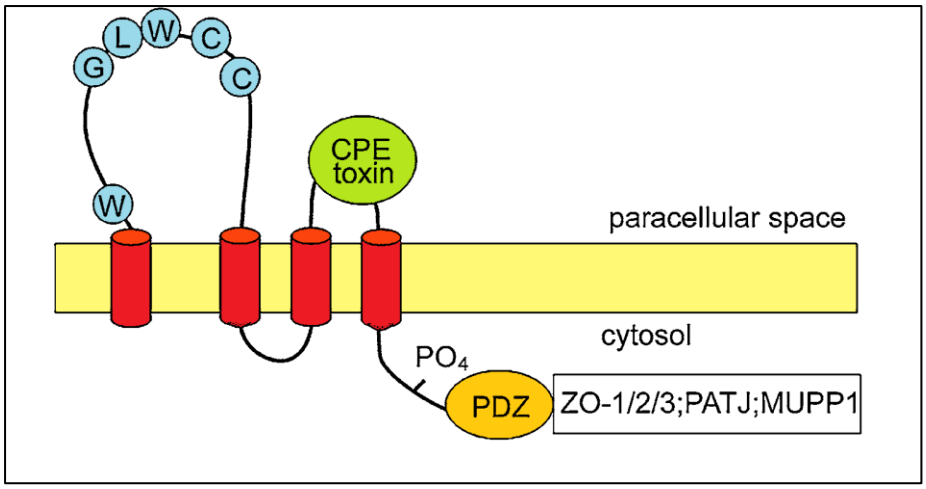



**Fig. 2 - Schematic representation of CLDN-1**

Recent studies confirm that CLDN-1 is required for late step of HCV entry in the post-binding phase of infection, subsequent to HCV binding to CD81 and SR-BI (9). Indeed the expression of CLDN-1 confers susceptibly to HCVpp infection in non-hepatic cell lines such as HEK t293 and SW13. Moreover, silencing of CLDN-1 inhibits HCV infection in susceptible cells (Huh7.5) (9).

Other studies indicate that distribution of CLDN-1 in tight junctions correlates with permissiveness to HCV infection (10, 11), thereby confirming that localization of CLDN-1 at tight junctions is critical for viral entry and HCV cellular tropism. In fact, while viral cell entry is undoubtedly essential for initiation of infection, direct cell-cell transmission (in which it is involved CLDN-1) probably constitutes the dominant mechanism of viral spread and persistence of infection (12, 13).

### *1.2.HCV Epidemiology and therapy*

Direct contact with infected blood and blood products, sexual relationships and use of injectable drugs are the main ways of HCV infection. Recent estimates indicate that 500.000 deaths are associated with HCV infection worldwide (14). The major burden of HCV infection derives from chronic infection, as 3-4 million people are newly infected each year and 170 million people are chronically infected (15) with the risk of developing liver disease including cirrhosis, fibrosis and liver carcinoma (50-76% of all liver cancers) which are the prime reason for liver transplants.

In the next future, the proportion of all US HCV cases with liver cirrhosis is estimated to become even larger than the current levels (16), thus resulting in an increased need for liver transplantation.

The mechanism by which HCV escapes the immune responses and establishes a chronic infection are not completely defined. HCV replicates at high rate (17) and its RNA-dependent RNA polymerase lacks proofreading capacity. This results in high genetic variability, which allows for rapid evolution under selection either by the immune system or in the presence of antiviral drugs. Thus, the emergence of resistance is the major hurdle that needs to be overcome in developing new therapeutic approaches against HCV.

The current standard of care, i.e. a combination of Pegylated-Interferon alpha and Ribavirin (PegIFN/RBV) is effective in only about 50% of patients and is often poorly tolerated (18). There is an acute need of new therapies especially for patients that undergo liver transplantation (LT) for HCV-related liver disease. Almost all of these patients suffer from graft re-infection, they are difficult to treat, respond less well and it is often necessary to wait several months after LT before starting therapy (19).

Although new anti-viral drugs targeting the virus protease or polymerase, such as telaprevir or boceprevir in clinical use since 2011, have been either approved or in clinical development, the available pre-clinical and clinical data already indicate that side toxic effects of the individual compounds and drug-resistant variants emerge during treatment (20 - 24).

Thus, antibodies that are capable of preventing HCV infection are an attractive novel therapy based on a different mechanism of action. There are at least two major therapeutic settings that would greatly benefit from an effective antibody based therapy:

1) in chronically infected patients: in combination with PegIFN/RBV to increase the number of responders, or in combination with novel anti-viral drugs to prevent re-infection by drug-resistant mutants;

2) in patients undergoing LT: as a monotherapy during the anhepatic phase and during the first days/weeks following transplantation to prevent re-infection of the graft; in combination with PegIFN/RBV, during the anhepatic phase and during the first days/weeks following transplantation to increase the number of responders.

HCV displays a very high inter- and intra-individual genetic variability especially in the E1 and E2 envelope glycoproteins where neutralizing determinants are present. Therefore antiviral neutralizing antibodies are effective at best against only a small number of viral variants (15). Accordingly, passive immunotherapy in LT patients with polyclonal or monoclonal antibodies against the viral envelope can only transiently lower RNA levels, but do not prevent re-infection presumably due to the large

heterogeneity in the infecting viral population (25, 26). In addition, it was shown that HDL can reduce the neutralizing effect of anti-HCV antibodies (27, 28) raising additional concerns about the efficacy of anti-HCV antibodies for active or passive immunotherapy.

For the reasons mentioned above, the recent discovery of cell surface molecules required for HCV viral entry offer a novel perspective for the development of new preventative and therapeutic agents, suggesting that viral cell entry could become a promising antiviral target complementary to targets of current standard of care and new antiviral drugs.

Four different cell surface receptors have been shown to be necessary for HCV infection in the hepatocyte: the tetraspannin CD81, the human scavenger receptor class B type I (SR-BI), the tight junction Claudin-1 and Occludin (29 - 33).

Antibodies against CD81 (of mouse origin) and SR-BI (of both mouse and human origin) have been generated and have shown to be capable of inhibiting HCV infection *in vitro* (a-CD81 and a-SR-BI) and in vivo (a-SR-BI) when used at very high doses (not feasible for their use for therapy in humans). Human monoclonal antibodies against Claudin and Occludin are not yet been reported, but some anti-CLDN-1 antibodies have been raised by genetic immunization of Wistar rats and found to efficiently inhibit infection by HCV of all major genotypes by blocking cell entry (34).

As a further progress in the field we describe here the selection by phage display on live cells (35, 49) of a panel of novel high affinity fully human monoclonal antibodies directed to Claudin-1 that could be used for prevention and therapy of HCV infection.

We show here that they recognize different epitopes and are endowed with anti-HCV inhibition activity when used either alone or in combination to prevent HCV infection in cell cultures of hepatocytes *in vitro*.

Such mAbs may represent interesting candidates for clinical development in HCV therapy of chronic infection and in post-transplant setting.

## *2. Human immunoagents for cancer immunotherapy*

Cancer is one of the most common diseases in developed countries with approximately 20% of deaths per year in the world (36). The absence of selectivity for tumor cells is the main limit of the conventional anti-cancer treatments, such as chemotherapy and radiotherapy. For this reason immunotherapy, based on the use of monoclonal antibodies (mAb) specific for tumor associated antigens (TAA) overexpressed on tumor cells, has been

shown to be an effective tool for the treatment of certain carcinomas. The transmembrane tyrosine kinase receptor ErbB2, overexpressed on tumor cells of different origin, such as breast, ovary, lung (37, 38) and gastric cancer, has been considered a very attractive target for cancer immunotherapy (39).

In particular, the overexpression of ErbB2 and its heterodimerization with ErbB3 on tumor cells, leads to the activation of the signaling pathways promoting cancer growth and survival, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase pathways (PI3K) (Fig. 3).

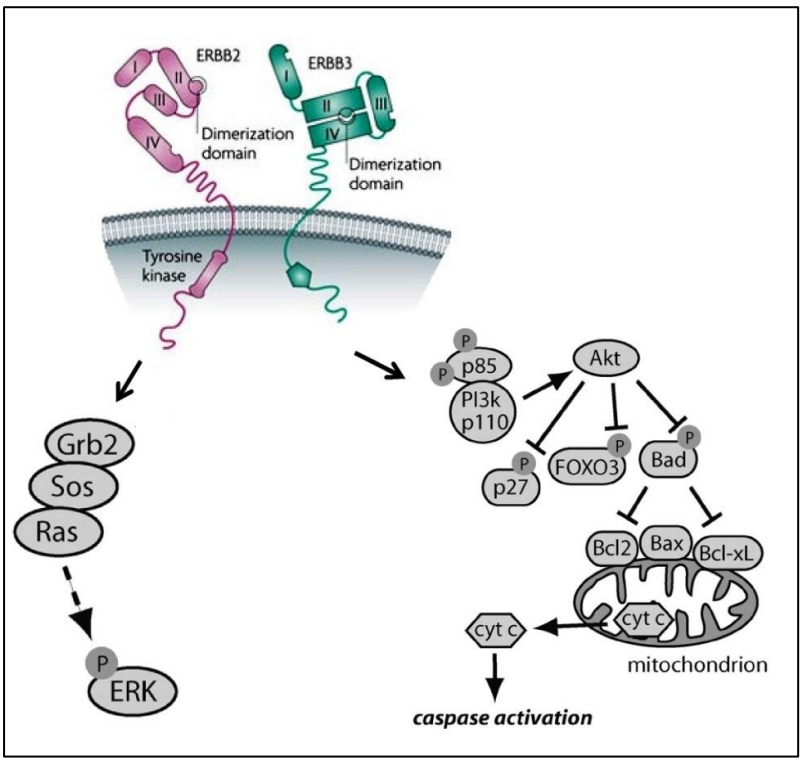



**Fig. 3 - ErbB2 signaling pathway**

Herceptin, a humanized monoclonal antibody widely used for immunotherapy of ErbB2-positive carcinomas (40) is the first ErbB2-targeted therapeutic immunoagent approved by the United States Food and Drug Administration. Herceptin is currently in clinical use with some success for breast cancer, but it may cause cardiotoxicity and a high fraction of patients develop resistance to Herceptin treatment (41 - 43).

Large-scale clinical studies have shown that 7 or 28% of patients suffer from cardiac dysfunction when Herceptin is used alone or in combination with anthracyclines, respectively (44 - 46).

The underlying mechanism of Herceptin cardiotoxicity is based on its capacity to inhibit the neuregulin (NRG-1) mediated heterodimerization of

ErbB2/ErbB4 on cardiac cells, thus interfering with the pathway of myocyte survival, induced by ErbB2 (47). To overcome the limits of Herceptin, novel anti-ErbB2 immunoagents have been developed in our laboratory by phage display technology. This methodology allows for the isolation of fully human antibody fragments, which are the minimal portion of an antibody capable of binding to the antigen. A single chain variable fragment (scFv) is made up of the variable domains of the heavy (VH) and light chains (VL) connected by a flexible oligopeptide. This fragment retains the binding specificity for the antigen but is able to diffuse more easily into the tumor masses.

With the aim of obtaining a novel fully human anti-ErbB2 antibody devoid of cardiotoxic side effects, a human ErbB2-specific scFv was isolated in our laboratory in collaboration with a research group of the *Imperial College* of London. This scFv, named Erbicin, binds to the cells overexpressing ErbB2, by recognizing a distinct epitope from that of Herceptin and pertuzumab (48); it is internalized through receptor mediated endocytosis, and selectively inhibits the proliferation of ErbB2-positive tumor cells (49).

Erbicin was fused with the Fc region of a human IgG1 (50) to obtain a construct, which was called *Erb-hcAb* for its compact size (100 kDa) if compared with the full size (155 kDa) of a natural IgG (Fig. 4). This construct should acquire the immuno-effector functions of the Fc domain while retaining the ability to diffuse more easily in the tumor masses due to its reduced molecular size with respect to a full size antibody.

*Erb-hcAb* selectively binds to breast tumor cells expressing ErbB2 and inhibits their growth in vitro and in vivo, with no effects on ErbB2-negative cells. Moreover, *Erb-hcAb* is endowed with both antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (49, 51). Interestingly, *Erb-hcAb* does not cause cardiotoxic effects, both in vitro on rat cardiomyoblast and human cardiomyocytes and in vivo in a mouse model, unlike Herceptin that it was shown to be strongly toxic (47, 52 - 54).

Infact Herceptin, in contrast to *Erb-hcAb*, induces apoptosis in cardiac cells and inhibits the ErbB2-ErbB4 heterodimerization mediated by Neuregulin-1 (47).

Furthermore, *Erb-hcAb* is active in vitro and in vivo against some ErbB2-positive breast cancer cell lines resistant to Herceptin (55).

To validate *Erb-hcAb* as a therapeutic agent, we analyzed its inhibitory effects also on gastric tumor cells expressing ErbB2, which have been reported to be less sensitive to Herceptin treatment (49, 50, 55).

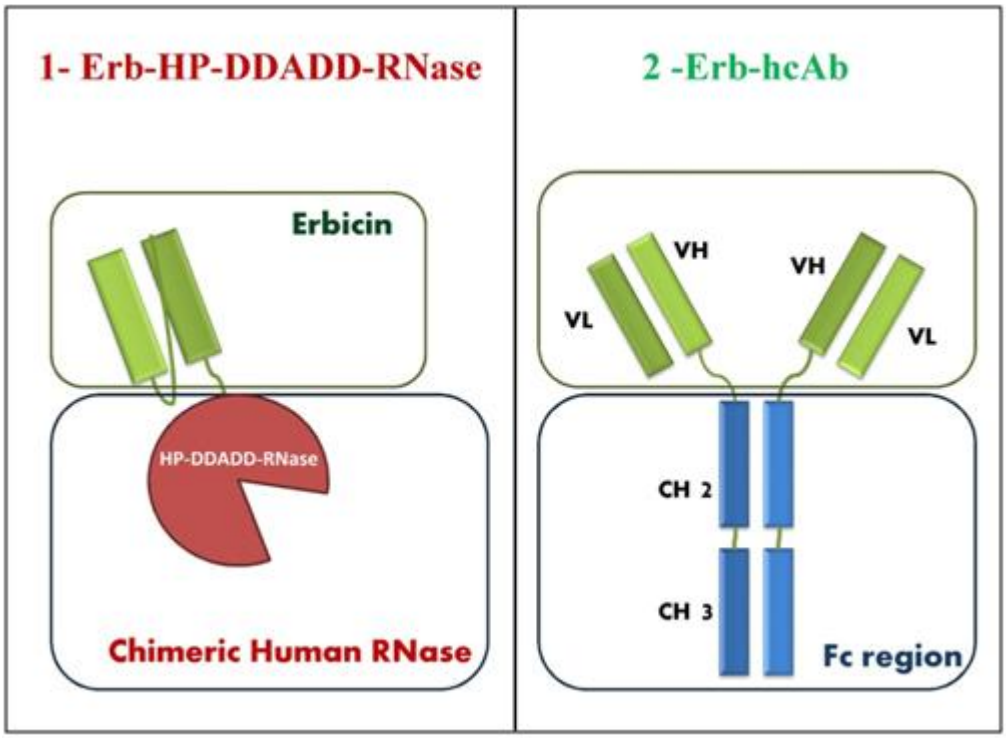



**Fig. 4 - Schematic representation of anti-ErbB2 immunoagents**

As an alternative strategy scFvs can be conjugated with radionuclides or toxins (56, 57), combining the potent toxicity of these molecules with the antigen specificity of antibodies (58). However, the so called "immunotoxins" could cause non-specific toxicities, such as vascular leak syndrome and hepatotoxicity, thus limiting the therapeutic potential of these immunoconjugates (59, 60).

To circumvent these problems, the toxin has been replaced by a non-toxic and non-immunogenic RNase, which becomes toxic only upon its internalization in the target cells mediated by the antibody moiety (61, 62).

In our laboratory a novel fully human immunoRNase, named ERB-HP-RNase, was obtained by fusing Erbicin with human pancreatic RNase (63).

This immunoconjugate retains both the Erbicin binding affinity to ErbB2-positive cells, and the enzymatic activity of native human pancreatic RNase. When tested in vitro and in vivo, ERB-HP-RNase was able to discriminate between target and non-target cells, and to specifically inhibit the proliferation of ErbB2-positive tumor cells either sensitive or resistant to Herceptin treatment (51, 55, 63).

However, ERB-HP-RNase was found to be sensitive to the neutralizing action of the cytosolic RNase inhibitor (RI), a 50 kDa monomeric protein present in the cytosol of mammalian cells, which inhibits the ribonucleolytic activity of all monomeric members of the human pancreatic RNase family (64 - 66).

Thus, to develop a more potent immunoRNase, Erbicin was fused with an inhibitor-resistant variant of human pancreatic RNase.

The variant RNase was designed to suppress, through Coulombic repulsion, its binding to RI, by the replacement of 5 aminoacidic residues crucial for the formation and the stability of the RI-RNase complex (R39D/N67D/N88A/G89D/R91D) (67).

The chimeric protein, named ERB-HP-DDADD-RNase (Fig. 4), was found to retain the high binding affinity to ErbB2-positive cells of Erbicin, as well as the enzymatic activity of the RNase moiety (68). Here we report that ERB-HP-DDADD-RNase is capable to discriminate between target and non-target cells, and to specifically inhibit the proliferation of ErbB2-positive cells with a more potent effect than the parental IR. Its antitumor potential has also been investigated *in vivo* in mice implanted with ErbB2-positive tumors resistant to Herceptin treatment.

# MATERIALS AND METHODS

### *1. Cell Cultures*

The eukaryotic Human Embryonic Kidney HEK t293, HEK 293 EBNA, the Huh-7.5 human hepato cellular carcinoma and Huh-7 human hepatoma cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM, Life Technologies, Inc., Paisley, U.K.) with the addition of non essential aminoacid solution (Gibco, Life Technologies, Inc., Paisley, U.K.). The HEK t293 cells transduced with the gene encoding Claudin-1 were grown in DMEM containing Blasticidin (2 µg/ml) (Gibco, Life Technologies, Inc.). The Chinese hamster ovary (CHO) cells (American Type Culture Collection, Rockville, MD, USA) were cultured in F12 medium (Gibco, Life Technologies, Inc.).

The human breast cancer SKBR3 cell line, the human epidermoid carcinoma A431 cell line and the human gastric cancer cell lines (NCI-N87, MKN-7, MKN-45 and AGS) were cultured in RPMI medium (Gibco, Life Technologies, Inc.). The KPL4 cell line from the malignant pleural effusion of a breast cancer patient with an inflammatory skin metastasis, and the JIMT-1 cell line established from a pleural metastatis of a 62-year-old breast cancer patient resistant to Herceptin were grown in DMEM (Gibco).

Media were supplemented with 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin (All from Gibco, Life Technologies, Inc.).

### *2. Antibodies*

The following antibodies were used: mouse HRP-conjugated anti-M13 monoclonal antibody (GE Healtcare Bio-Sciences AB, Uppsala, Sweden); mouse HRP-conjugated anti-c-myc-tag monoclonal antibody (Miltenyi Biotec Inc., Auburn, U.S.A.); mouse anti-Claudin-1 (C-terminal end) monoclonal antibody (Life Technology, Inc.); rabbit anti-loop 1 of Claudin-1 polyclonal antibody, (Abcam, Cambridge, U.K.); goat HRP-conjugated anti-Human IgG (Promega Corporation Madison, U.S.A.); goat HRP-conjugated anti-human Fc monoclonal antibody (Immuno Reagents, Inc., Raleight U.S.A.); mouse HRP-conjugated anti-His monoclonal antibody (Qiagen GmnH, Hilden, Germany), rabbit anti-HER2 monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-actin polyclonal antibody (Sigma-Aldrich), rabbit HRP-conjugated anti-immunoglobulins from goat antiserum (Thermo Scientific, Rockford, IL, USA); rabbit anti-Akt antibody, rabbit anti-phospho-Akt antibody, rabbit anti-p44/42 MAPK (Erk1/2) antibody, rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (All from Cell Signaling Technology, Inc.).

### *3. Bacterial Culture Media*

2xTY and LB (Luria-Bertani) culture media were used for the growth of bacteria. These media were prepared as described by Sambrook et al. (69).

### *4. Bacterial Strains*

For the production of the scFv expressed as a fusion with pIII protein of the phage, *E.Coli TG1* bacterial strain was used; for expression of the scFv as a soluble protein, *E.Coli SF110* bacterial strain was used.

The *E. coli* DH5α (Invitrogen, Life Technologies, Paisley, UK) and BL21 DE3 (Novagen-Merck Millipore, Darmstadt, Germany) strains were used for cDNA amplification and expression of immunoRNase, respectively.

### *5. Antibiotics*

The Antibiotics used were Ampicillin (100 µg/ml) and Kanamicin (25 µg/ml).

### *6. Preparation of Phage Particles from the Phagemid library*

Phagemid particles were rescued with M13-K07 from the library, as described previously (70). Briefly, the phage library was grown in 200 ml of 2xTY containing 100 µg/ml of Ampicillin and 1% glucose, until the optical density at 600nm (OD600) was 0.5. Subsequently, 1 x $10^9$ plaque-forming units of M13-K07 helper phage encoding trypsin-cleavable pIII, were added to 25 ml of bacterial culture and incubated for 1 hour at 37°C without shaking. Then the cells were centrifuged at 4000 rpm/min for 15 min, resuspended in 500 ml of 2xTY containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin and grown overnight by shaking at 30°C. Phage particles were purified and concentrated by two steps of polyethylene glycol (PEG) precipitation (69) and washed with 20 ml of sterile water. After an additional PEG-precipitation step, phages were resuspended in PBS, centrifuged at 12,000 rpm for 15 min, and stored at 4°C until use.

### *7. Selection of scFv-Phages on live cells*

The human Huh-7.5 cell line, naturally expressing high levels of Claudin 1, the HEK t293 cells untrasduced or trasduced with Claudin-1 and the CHO cells untrasfected or trasfected with a vector encoding Claudin-1 were detached with the cell dissociation solution (Sigma-Aldrich, Saint Louis, U.S.A.) and washed twice with PBS. For each round of panning, phages ($10^{13}$ cfu) were blocked with 5% milk powder (Sigma-Aldrich) in PBS for 15 minutes. The blocked phages were submitted to two following rounds of negative selection by two successive incubations with CLDN-1-negative cells (1 x $10^6$), carried out by gently rotating the suspension for 2

hours at 4°C. The unbound phages, collected by centrifugation at 1200 rpm for 10 minutes, were then used for the positive selection performed by using CLDN-1 trasduced HEK t293 ($1 \times 10^6$), or Huh-7.5 cells ($1 \times 10^6$), incubated for 16 hours by gently rotating at 4 °C. Cells were then recovered by centrifugation at 1200 rpm/min for 10 minutes and washed twice with PBS. The positive selection on CLDN-1 trasfected CHO cells was carried out by incubating the phages with of $2 \times 10^6$ adherent cells. After extensive washes with PBS, bound phages were eluted from positive cells with a solution of 1 µg/ml of Trypsin (Sigma-Aldrich), which was then stopped by the addition of complete EDTA-free protease inhibitor (Roche Diagnostic, Mannheim, Germany). The recovered phages were amplified by infecting *E.Coli TG1* cells to prepare phages for the next round of selection.

### *8. Preparation of scFv-phages for cell ELISA*

A TG1 culture was infected with the eluted phages (after 3-4 rounds of panning) and plated on 2xTY/agar containing 1% glucose and ampicillin (100 µg/ml). Individual clones were picked, transferred into 96-well plates and grown in 100 µl of 2xTY medium containing 1% glucose and ampicillin (100 µg/ml) for 18 hours in agitation at 37°C. Glycerol (20 %) was added to each well of these master plates before storage at -80°C. Replicas of the master plates were prepared and the scFv-phages were produced by superinfection with M13-K07 helper phage encoding trypsin-cleavable pIII as described above. The plates were centrifuged at 1200 rpm for 30 minutes at 4°C to pellet the bacteria, and aliquots of 50 µl of scFv-phage-containing supernatants were used for cell ELISA.

### *9. Analysis of positive clones*

Positive clones selected by cell ELISA were inoculated in 10 ml of 2xTY containing 100 µg/ml of Ampicillin and 1% glucose and grown up over night by shaking at 37°C to prepare the plasmids by using *QIAprep Spin Miniprep Kit* (QIAGEN, Venlo, Limburg, Netherlands). To determine the number of individual selected clones, the cDNA encoding the single chain variable fragment of positive clones was amplified by PCR from pCANTAB or pHEN2 plasmid, using the following forward primers: 5'-TTATTATTCGCAATTCCTTTAGTT-3' and 5'-ATGAAATACCTATTGCCTACG

GCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATG GCC-3' respectively, both complementary to the *pel B* leader sequence, located upstream the heavy variable region (VH) sequence, and a reverse primer 5'-CTCTTCTGAGATGAGTTTTTG-3', *Mycseq,* complementary to the sequence located downstream to the c-myc tag peptide. Reactions were performed with Taq DNA polymerase (*Expand High Fidelity PCR System*,

Roche Diagnostic) in a volume of 50 µl for 35 cycles under the following conditions: 45 seconds denaturating at 95°C, 30 seconds annealing at 60°C and 45 seconds extension at 72°C. The amplified products were analyzed by electrophoresis on 1% agarose gel. The nucleotide sequences encoding scFvs were determined by using the same PCR primers by an external facility (Bio-Fab Research srl, Rome, Italy). The sequences were analyzed with a software for DNA and amino acid editing and alignments (*GENtle-software*, Magnus Manske, University of Cologne, Germany).

### *10. Soluble scFv expression and Purification*

Cultures of *E. Coli SF110* infected with each of the anti-CLDN-1 scFv-phage preparation were grown in 200 ml of 2xTY medium containing 100 µg/ml ampicillin, until an absorbency of 0,8 optical density was reached. The expression of soluble scFv was induced by the addition of IPTG (1mM) (AppliChem GmbH, Darmstadt, Germany) to cell culture, which was then grown by shaking at room temperature for 16 hours. Cells were harvested by centrifugation at 4000 rpm for 20 minutes and resuspended in *B-PER buffer* (Pierce, Rockford, IL) for 20 minutes by gently rotation at room temperature. Cell suspensions were then centrifuged at 12000 rpm for 30 minutes at 4°C to obtain the periplasmic extract. Soluble scFvs were purified on immobilized-metal affinity chromatography, by incubating the periplasmic extract with Ni-NTA agarose (QIAGEN) for 2 hours by gently rotation. After extensive washes with PBS containing imidazole (20 mM) , the protein was eluted in $NaH_2PO_4$ buffer (50 mM, pH 8.0) containing NaCl (0.3 M) and imidazole (250 mM). The purity of the final preparation was evaluated by SDS-PAGE followed by Coomassie staining. Purified scFvs, analyzed also by Western blotting, were detected by using a mouse HRP-conjugated anti-c-myc-tag monoclonal antibody (Miltenyi Biotec Inc.).

### *11. Production and Purification of mAbs*

For the conversion of the selected scFvs into mAbs *In-Fusion™ HD cloning kit* (Clontech Laboratories, Inc. A Takara Bio Company, Mountain View, CA, U.S.A.) was used. This technology fuses DNA fragments, e.g. PCR-generated sequences and linearized vectors, efficiently and precisely by recognizing a 15 bp overlap at their ends. This 15 bp overlap can be engineered by designing primers for amplification of the desired sequences. To this aim appropriate primers were designed either complementary to the ends of the linearized vector (restriction sites are underlined) or to those of the variable-region-fragment sequences with 15 bp extensions homologous to vector ends.

*mAb A2 primers:*
VH FOR 5'-CTCTCCACAGGCGCGCACTCCCAGGTGCAGCTGGTGCAGT-3'
VH REV 5'-GGCATTGGGTGGATCCGTCGACAGGACTCACCCACGGTGACCAGGGTACCTTGGCCCC-3'
VL FOR 5'-CTCCACAGGCGTGCACTCCGAGCTGACTCAGGACCCTGCT-3'
VL REV 5'-TTCTGACTCACCTAGGACGGTCAGCTTGGTCCCTCCGCCGAA-3'

*mAb B9x primers:*
VH FOR 5'-CTCTCCACAGGCGCGCACTCCGAGGTGCAGCTGTTGGAGT-3'
VH REV 5'-GGCATTGGGTGGATCCGTCGACAGGACTCACCACTCGAGACGGTGACCATTGTCCC-3'
VL FOR 5'-CTCCACAGGCGTGCACTCCCTTTCTTCTGAGCTGACTCAGGAC-3'
VL REV 5'-TTCTGACTCACCTAGGACGGTGACCTTGGTCCCTCC-3'

*mAb D10x primers:*
VH FOR 5'-CTCTCCACAGGCGCGCACTCCGAGGTGCAGCTGTTGGAGT-3'
VH REV 5'-GGCATTGGGTGGATCCGTCGACAGGACTCACCACTCGAGACGGTGACCATTGTCCC-3'
VL FOR 5'-CTCCACAGGCGTGCACTCCCAATCTGCCCTGACTCAGCCT-3'
VL REV 5'-TTCTGACTCACCTAGGACGGTCAGCTTGGTCCCTCC-3'

*mAb H9C1 primers:*
VH FOR 5'-CTCTCCACAGGCGCGCACTCCGAGGTGCAGCTGTTGGAGT-3'
VH REV 5'-GGCATTGGGTGGATCCGTCGACAGGACTCACCACTCGAGACGGTGACCATTGTCCC-3'
VL FOR 5'-CTCCACAGGCGTGCACTCCCTTTCTTCTGAGCTGACTCAGGAC-3'
VL REV 5'-TTCTGACTCACCTAGGACGGTGACCTTGGTCCCTCC-3'

*mAb F7C2 primers:*
VH FOR 5'-CTCTCCACAGGCGCGCACTCCGAGGTGCAGCTGTTGGAGT-3'
VH REV 5'-GGCATTGGGTGGATCCGTCGACAGGACTCACCACTCGAGACGGTGACCAGGGTGCC-3'
VL FOR 5'-CTCCACAGGCGTGCACTCCCAGTCTGTGCTGACTCAGCCA-3'
VL REV 5'-TTCTGACTCACCTAGGACTAGTACGGTTCAGCTTTGTTCCTC-3'

*mAb E9C2 primers:*
VH FOR 5'-CTCTCCACAGGCGCGCACTCCGAGGTGCAGCTGTCGGAGT-3'
VH REV 5'-GGCATTGGGTGGATCCGTCGACAGGACTCACCACTCGAGACGGTGACCATTGTCCC-3'
VL FOR 5'-CTCCACAGGCGTGCACTCCCTTGAAATTGTGTTGACGCAGTCT-3'
VL REV 5'-TTCTGACTCACCTAGGACTTTAATCTCCAGTCGTGTCCCTTG-3'

PCR reaction products were purified by 1% agarose gel by *QIAquick Gel Extraction Kit* (QIAgen) and inserted in the appropriate vectors by using the *in-Fusion HD Enzyme Premix*, according to the manufacturer's reccomendations. The VH sequences were cloned in the linearized PEU 8.2 vector, previously digested by using BamH1 and BssHII restriction endonucleases; VL sequences were cloned in linearized PEU 4.2 vector, previously digested by using ApaL1 and AvrII restriction endonucleases. A sequence analysis was then performed to confirm the successful cloning of the inserted sequence with the correct reading frame.

Aliquots of 100 µg of the recombinant vectors encoding each VH and VL pair were co-transfected in *HEK 293-EBNA* by using Lipofectamine® Transfection Reagent (Life Technologies, Inc.) and grown up for 10 days in 150 mm tissue culture dish in a humidified incubator at 37°C with 5% $CO_2$. The antibodies secreted in the medium were purified by using *HiTrap Protein-A HP* (GE Healthcare life sciences, New York, U.S.A.) equilibrated in a phosphate-based buffer, pH 8.0 (binding buffer). After extensive washes,

the bound antibodies were eluted in a Tris-Glycine buffer, pH 2.5 and readily neutralized with the addition of 1 M Tris solution at pH 8.0. The concentration of the purified antibodies were determined by *BCA Protein Assay* kit (Pierce, Thermo Fisher Scientific). The purity of the final preparations was evaluated by SDS-PAGE and following staining with Coomassie blue solution (20% isopropanol, 10% acetic acid, 0.1% Coomassie blue).

***12. Cell ELISA assay***

To confirm the binding specificity for Claudin-1 of the selected scFv-phages or purified mAbs, cell ELISA analyses were carried out by using CLDN-1-positive and -negative cells. Briefly, the cells were detached with non-enzymatic cell dissociation solution (Sigma-Aldrich), washed and resuspended in PBS/BSA 6% and plated in 96 multiwell plates ($2 \times 10^5$ cells for each well).

The scFv-phages or mAbs were then added and incubated in PBS/BSA 3% for 90 minutes in agitation at room temperature. For the binding detection, cells were incubated for 1 hour either with 100 µl of PBS buffer containing 4% FBS and mouse HRP-conjugated anti-M13 monoclonal antibody (1:5000 dilution) for scFv-phage detection, or with 100 µl of PBS buffer containing 3% BSA and goat HRP-conjugated anti-human IgG (1:2500 dilution, Promega) for mAbs detection. After 3 washes with PBS, cells were finally resuspended in 50 µl of TMB reagent (Sigma-Aldrich) and incubated for 2 min before stopping the reaction with 50 µl of 1 N HCl. The absorbance ($A_{450}$) was measured by using a VICTOR X3 Multilabel Plate Reader (PerkinElmer) and the data were analyzed by using a spreadsheet program (Microsoft Office Excel).

ELISA assays for the immunoRNases ware performed on ErbB2-positive SKBR3, KPL4 and JIMT-1 breast cancer cells, NCI-N87, MKN7 and AGS gastric tumor cells and ErbB2-negative A431 control cells incubated in the absence or in the presence of increasing concentrations (2,5 nM, 5 nM, 10 nM, 25 nM or 50nM) of each immunoRNase (ERB-HP-RNase or ERB-HP-DDADD-RNase) as described above.

ELISA assays with Erb-hcAb were carried out on ErbB2-positive gastric tumor cells mentioned above, incubated with increasing concentrations of the antibody. Washes and incubations with the secondary antibody were performed as described above (50).

For detection of the IR or the human antibodies, a mouse HRP-conjugated anti-His monoclonal antibody (Qiagen) or a goat HRP-conjugated anti-human Fc monoclonal antibody (Immuno Reagents, Inc.) were used, respectively.

### *13. Competitive Cell ELISA*

To determine whether the novel mAbs recognize different Claudin-1 epitopes, competition experiments were carried out by cell ELISA. Positive CLDN-1 cells, detached with non-enzymatic cell dissociation solution (Sigma-Aldrich) were blocked in PBS/BSA 6%, plated in a 96 multiwell plate (1 x $10^5$ cells for each well) and incubated for 90 minutes with 200 nM of each mAb in PBS/BSA 3% in agitation at room temperature. Then the cells were resuspended in 100 µl of each scFv-phage (1 x $10^{11}$ cfu) prepared in a PBS/milk 2,5 % solution and incubated for further 2 hours in agitation at room temperature. For the detection of bound phages, cells were incubated for 1 hour with a mouse HRP-conjugated anti-M13 monoclonal antibody (GE Healtcare). After extensive washes, cells were resuspended and analyzed as described above.

### *14. Cell lysis, Immunoprecipitation and Western Blot Analyses for anti-CLDN-1 mAbs*

Claudin-1 positive cells (2,5 x $10^7$), detached with non-enzymatic cell dissociation solution (Sigma-Aldrich), were resuspended in 1,5 ml of lysis buffer [10 mM of Tris HCl pH 7.4, 150 mM NaCl and 1% NP40] containing complete EDTA-free protease inhibitor (Roche Diagnostic). After 45 minutes at 0°C the extracts were clarified by centrifugation at 12000 rpm for 20 minutes. Each cell lysate was incubated with 20 µl of Protein-A Sepharose (Sigma-Aldrich) for 1 hour by gently rotation at 4°C. After a centrifugation at 2000 rpm for 5 minutes, the supernatant was incubated at 4°C for 16 hours by gently rotation with 5 µg of mouse anti-C-terminal region of Claudin-1 monoclonal antibody (Life Technology, Inc.), 5 µg of rabbit anti-loop 1 of Claudin-1 polyclonal antibody, (Abcam) or 10 µg of each mAb selected by phage display. Each immune-complex was then collected by adsorption to Protein-A Sepharose for 1 hour at 4°C. After four washes and a centrifugation at 2000 rpm for 5 minutes, the proteins released by boiling in loading buffer (71) were loaded on 7,5% SDS-PAGE and electroblotted onto Nitrocellulose Transfer Membrane (Whatman GmbH, Dassel, Germany). The Claudin-1 protein was detected by using a mouse anti-C-terminal region of Claudin-1 monoclonal antibody (Life Technology, Inc.), as previously described (72).

### *15. HCVcc generation and neutralization assays*

All chimeras were produced as previously described (73). Neutralization assays were performed by seeding Huh 7.5 cells (1.5 × $10^5$) in a 96-well plate (BD Biosciences). After 24 h, each monoclonal antibody was added at the stated concentrations to cells, incubated for 1 h at 37°C in a humidified atmosphere containing 5% $CO_2$, and washed twice with PBS. Virus was then

added and incubated at 37°C in a humidified atmosphere containing 5% CO2, for 72 h. Percentage of infected cells in the presence of antibodies was determined by comparison to the number of cells infected in the absence of inhibitors, as determined by NS5A staining (74).

***16. Cell viability and Cytotoxicity assays***

Huh-7.5 cells were seeded in 96-well flat-bottom plates at a density of 1 x $10^4$/well and incubated in a humidified incubator at 37°C with 5% $CO_2$ for 16 hours. After the addition of 100 or 500 nM of mAbs in the culture medium, cells were incubated for 72 hours in the same conditions. Viable cells were counted by the trypan blue exclusion test at suitable time intervals. Cell survival was expressed as percent of viable cells in the presence of the monoclonal antibody under test with respect to negative control cultures grown in the absence of the protein. Typically, cell survival values were obtained from at least three independent experiments in which triplicate counts were determined; standard deviations were less than 5 %. Cell lysis was determined by measuring the release of lactate dehydrogenase (LDH) using a LDH detection kit (Roche Diagnostic). Lysis was calculated as the percent of cytolysis measured in the presence of each mAb, taking as 100% the maximal LDH release determined by lysis of target cells treated with 1% Triton X-100.

***17. Expression and purification of ERB-HP-DDADD-RNase***

Cultures of *E. coli* BL21 DE3, previously transformed with the recombinant pET22b+ expression vector, were grown at 37°C in LB medium containing 50 µg/ml ampicillin until the exponential phase was reached. For the expression of soluble IR, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Applichem GmbH) was added to the cell culture, which then was grown at room temperature for 4 h. The cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C.

For the purification of the protein, the periplasmic extract, obtained by resuspending the bacterial pellet in B-PER® buffer (Pierce, Thermo Fisher Scientific, Rockford, IL, USA), was loaded on an immobilized-metal affinity chromatography (IMAC) by incubation with cobalt-chelating resin (TALON®; Clontech, Palo Alto, CA) at 25°C for 2h by gently rotation. After extensive washes in an appropriate buffer (PBS, 0.16 M NaCl, 20 mM imidazole), the elution step was performed in the same buffer containing a higher concentration of imidazole (250 mM). The concentration of the purified protein was determined by *BCA Protein Assay* kit (Pierce, Thermo Fisher Scientific) and the purity of the final preparations was evaluated by SDS-PAGE and following staining with Coomassie blue solution (20%

isopropanol, 10% acetic acid, 0.1% Coomassie blue) and by western blotting analyses with a mouse HRP-conjugated anti-His antibody as described above.

***18. RNase activity and inhibition assays***

RNase activity was tested by the acid-insoluble RNA precipitation assay as described previously (75) on yeast RNA (8 mg/ml). Yeast RNA was incubated with the enzyme under test in the reaction buffer (50 mM Tris-HCl, pH 8.0 containing 0.15 M NaCl) at 37°C for 30 min. The reaction was stopped and undegraded RNA was precipitated by addition of 10% cold perchloric acid containing 0.25% uranyl acetate. After incubation on ice for 15 min, the samples were centrifuged at 12000 rpm for 15 min at 4°C and the absorbance at 260 nm of the supernatant was determined. In this assay one unit of enzymatic activity is defined as the amount of enzyme that generates an increase of 1 OD at 260 nm.

For the inhibition assays, appropriate amounts of ERB-HP-RNase or ERB-HP-DDADD-RNase were pre-incubated with increasing concentrations of RI (Promega) in the presence of 2mM DTT at 37°C for 10 min before starting the activity test as described above (76).

***19. Cellular internalization assays***

ErbB2-positive SKBR3 cells and JIMT1 cells resistant to Herceptin were seeded in 6-well flat-bottom plates at a density of 6 x $10^5$/well and grown at 37°C for 24 h in culture medium. After the addition of an immunoRNase at a concentration of 200 nM, cells were incubated for 2 h at 37°C. Cells were then washed extensively with a stripping solution of 50 mM glycine buffer, pH 3.0, containing NaCl (1.0 M), harvested with cell dissociation solution and treated as previously described (Borriello et al., 2011). Internalized Erb–HP-RNase or Erb–HP-DDADD-RNase was detected by using appropriate dilutions of the anti-HPRNase antibody, followed by a HRP-conjugated anti-rabbit antibody. The signal intensity of the reactive bands was measured with a phosphorimager (45–710; Bio-Rad, Hercules, CA, USA).

***20. Effects of Erb-hcAb on tumor cell growth***

Gastric tumor cells were seeded in 96-well, flat-bottom plates at a density of 7.5 x $10^3$/well. After addition of the protein under test, viable cells were counted by the trypan blue exclusion test at suitable time intervals. Cell survival was expressed as percent of viable cells in the presence of the monoclonal antibody under test with respect to negative control cultures grown in the absence of the protein.

Growth inhibition of gastric tumor cells was assessed by using the MTS assay (Promega) method based on the bioreduction of 3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by cells to a formazan product that is soluble in tissue culture media and can be detected spectrophotometrically. For this assay, cells were seeded in 96-well plates in complete growth media. After 24 h, cell culture media were replaced with media containing 0.1 % fetal bovine serum (FBS) in the absence or in the presence of Erb-hcAb, which was added at concentrations ranging from 2 to 500 nM. At day 1, 2, or 3, aliquots of MTS (Promega) (20 µl) were added to each well and plates were incubated at 37°C for 2–3 h. The reaction was monitored by measuring Absorbance at 490nm.

### *21. Effects of ERB-HP-DDADD-RNase on tumor cell growth*

Cells were seeded in 96-well flat bottom plates, ErbB2-positive SKBR3 control cells at a density of 1.5 x $10^4$/well, Herceptin-resistant JIMT-1 and A431 cells at 5 x $10^3$/well, NCI-N87 and AGS cells at 1 x $10^4$/well, and MKN-7 cells at 2 x $10^4$/well. To test the effects of the immunoRNases on cell growth, SKBR3, A431, NCI-N87, MKN-7 and AGS cells were incubated at 37°C for 72 h in culture medium in the absence or presence of increasing concentrations (25–100 nM) of the immunoRNase. The cell counts were determined in triplicate using the trypan blue exclusion test.

### *22. In vivo antitumor activity of Erb-HP-DDADD-RNase*

The *in vivo* antitumor activity of Erb–HP-DDADD-RNase was determined in Balb/cAnNCrlBR athymic (nu/nu) mice (Charles River Laboratories, Calco, Italy). Herceptin resistant JIMT-1 cells were suspended in sterile PBS and injected subcutaneously into mice at a density of 2 x $10^6$ cells/mouse. On Day 30, when tumors were clearly detectable, Erb–HP-RNase or Erb–HP-DDADD-RNase was administered intraperitoneally five times at 72-h intervals to groups of 5 mice at doses of 1.2 mg $kg^{-1}$ of body weight. Another group of control mice was treated with identical volumes of sterile PBS to monitor tumor development. Tumor volume (V) was calculated by the formula of rotational ellipsoid: $V = A \times B^2/2$, where A is the axial diameter and B is the rotational diameter as measured with a caliper.

### *23. Effects of Erb-hcAb on ErbB2 Signaling pathway*

For the evaluation of the effects of Erb-hcAb on the expression and phosphorylation of proteins involved in the ErbB2 signaling pathway, a Western blotting analysis of lysates from untreated or treated NCI-N87 cells was performed. Detection was carried out by using the following antibodies: rabbit anti-Akt antibody, rabbit anti-phospho-Akt antibody, rabbit anti-p44/42 MAPK (Erk1/2) antibody, rabbit anti-Phospho-p44/42 MAPK

(Erk1/2) (Thr202/Tyr204) antibody (All from Cell Signaling Technology, Inc.).

### *24. ADCC tests*

NCI-N87 target cells were detached from culture dishes with a cell dissociation solution (Sigma), transferred to 96-well plates (7,000 cells/well), and incubated for 16 h at 37°C. Subsequently, PBMCs from a healthy donor were added at effector:target ratio 10:1 in the absence or presence of increasing concentrations of Erb-hcAb or Herceptin (5–200 nM) and incubated for 24–48 h at 37°C. Controls included target cells incubated in the absence of effector cells or in the presence of immunoagents alone. Tumor cell lysis was determined by measuring the release of lactate dehydrogenase (LDH) using a LDH detection kit (Roche Diagnostic) as described above.

### *25. In vivo antitumor activity of Erb-hcAb*

In vivo experiments were performed with 6-week-old female Balb/c nude mice (Charles River Laboratories, Calco, Italy). NCI-N87 cells ($2 \times 10^6$) were suspended in 0.2 ml sterile PBS and injected subcutaneously (day 0) in the right paw. At day 10, when tumors were clearly detectable, Erb-hcAb or trastuzumab dissolved in PBS was administered intraperitoneally to two different groups of six mice at doses of 5 mg/kg body weight, seven times, at 72-h intervals. Another group of control mice was treated with identical volumes of sterile PBS. During the period of treatment, tumor volumes (V) were measured with a caliper and calculated by the formula of rotational ellipsoid $V = A \times B^2 / 2$ (A is the axial diameter and B the rotational diameter). SD values were less than 10 %.

# RESULTS

## *1. Human antiviral immunoagents for antiviral therapy*

### 1.1. *Parallel selections of human scFvs on a panel of different Claudin-1-positive cells*

Phage particles from Phagemid library were prepared as described in Materials and Methods. The strategy used for the isolation of anti-Claudin-1 scFv-phages consisted in multiple selections with the use of different combinations of positive, *i.e.*, antigen-bearing, and -negative cell lines. This approach was devised to guarantee an efficient selection of a large number of clones with a high specificity for Claudin-1.

In the first strategy, human hepatoma cells Huh7.5, naturally expressing high levels of Claudin-1 (CLDN-1), were used as antigen-positive cells in the selection and HEK 293 cells lacking CLDN-1, were used in a subtractive step. In the second combination, HEK 293 cells trasduced with human Claudin-1 and untrasduced HEK 293 were used as antigen-positive and antigen-negative cells, respectively.

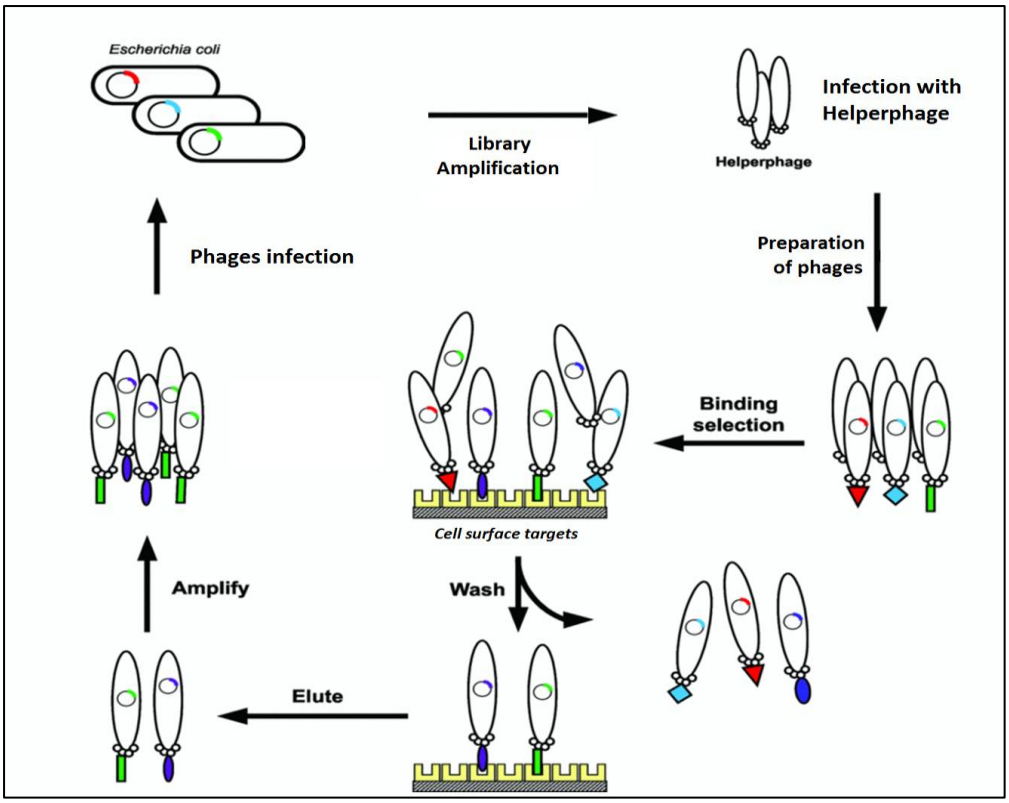



**Fig. 5 - Schematic representation of each selection round**

In the third, CHO cells transfected with the expression vector encoding Claudin-1 and untransfected CHO have been used as antigen-positive and -negative cells, respectively. In all the selections the negative cells have been used to deplete the library of phage antibodies that bind to common cell surface antigens.

In each selection round of the first two strategies, CLDN1-positive cells were incubated with the antibody phage display library ($10^{13}$ cfu/ selection) pretreated with the counterpart negative cells (untrasduced HEK 293), as described in the Methods. After 16 hours of incubation at 4°C, cells were washed, phage bound to the cell surface were displaced and used to infect *E. Coli* TG1 for amplification and further rounds of selections (Fig. 5).

Initially, two rounds of selection were performed either with Huh7.5 or with HEK t293-CLDN-1 cells in suspension. Positive phages, which selectively bound to a Claudin-1-positive cell line, were submitted to two additional rounds of selection, using either the same combination of cells or crossing the selection with the other one to further increase the chance to select phages with a high degree of specificity for CLDN-1 (see Fig. 6).

Strategy 3 was performed by panning the library directly on adherent CHO-CLDN-1 cells after the subtraction of non-specific clones on adherent untrasfected CHO cells, in order to select scFv against Claudin-1 in its natural environment within intercellular contacts.

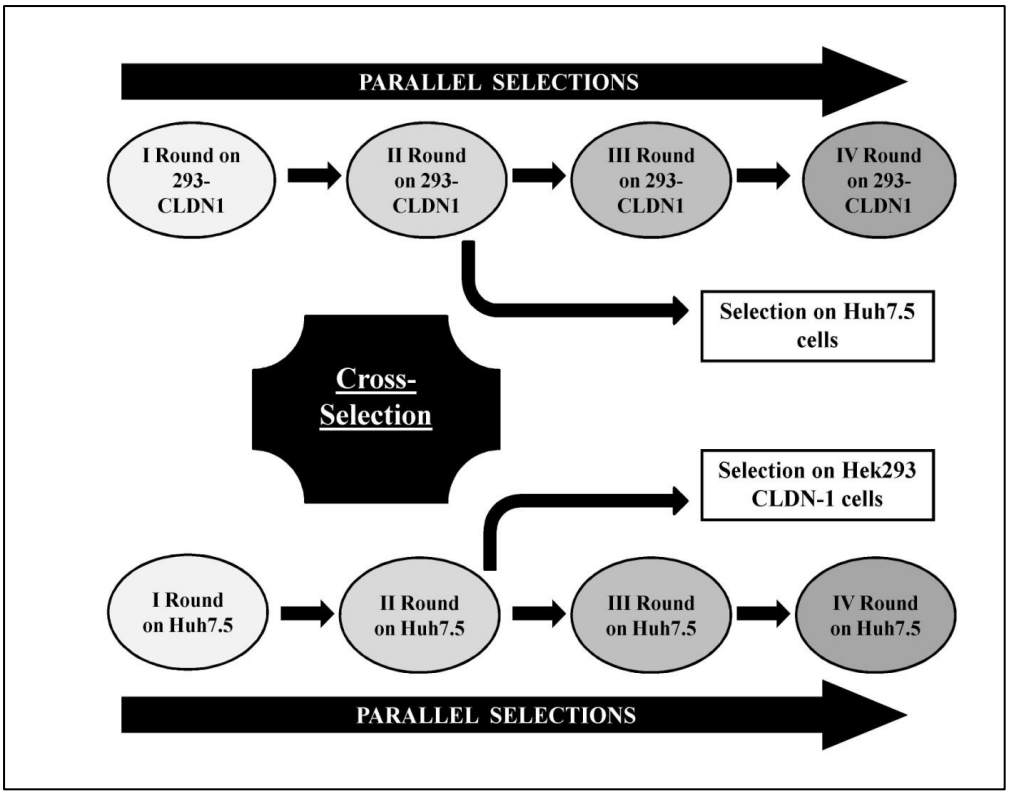



**Fig. 6 - Parallel and crossed selection on different cell lines**

### *1.2.Analysis of positive clones*

The strategy used for the analysis of positive clones and the following production of fully human antibodies is shown in figure 7.

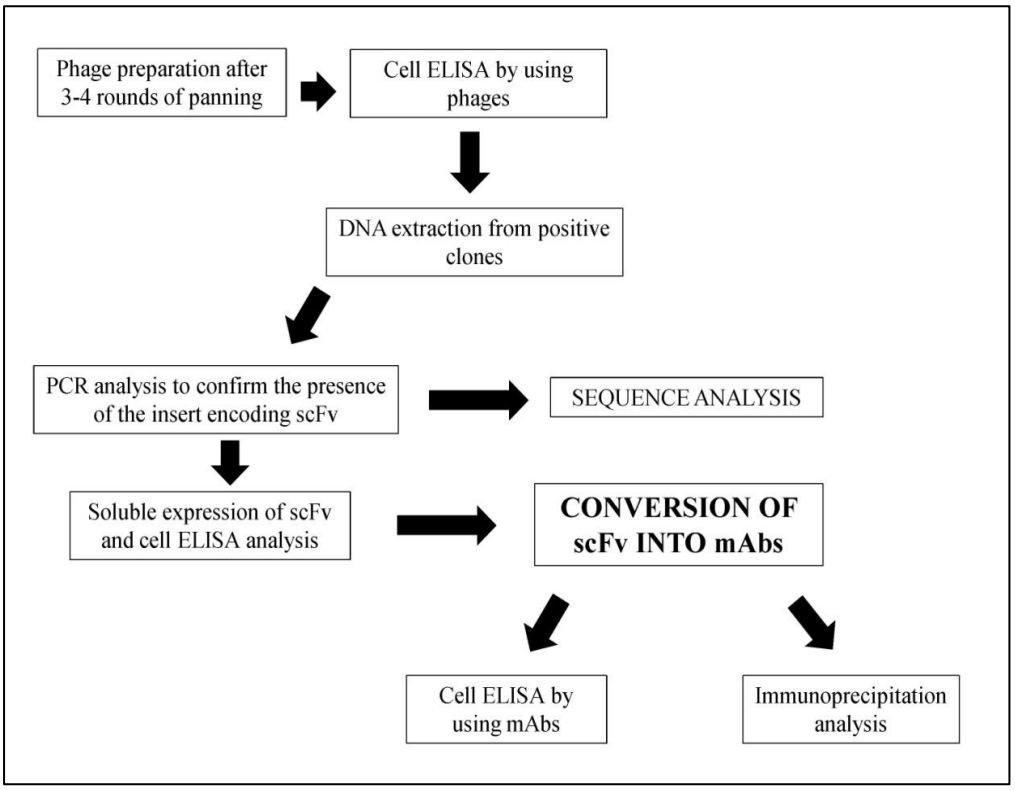
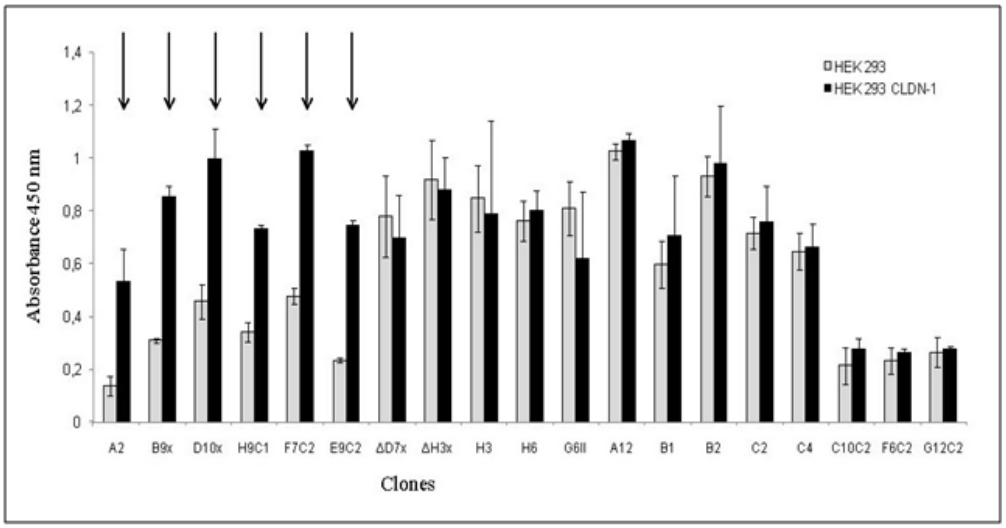



**Fig. 7 - Strategy for the clones analysis**

Briefly, cell ELISA assays on CLDN-1 negative and positive cells were carried out to identify Claudin-1-positive clones after 3-4 selection rounds of each selection strategy. As shown in Fig. 8-A, we selected those clones that were capable to discriminate between CLDN-1-positive and –negative cells. The positive clones (approximately 20%) were analyzed by PCR analysis to confirm the presence of the cDNA encoding the scFv (Fig. 8-B).

The DNA preparations from clones containing a specific CLDN-1-scFv were then sequenced by using the same primers already used for PCR (see Materials and Methods).

**A**




**B**

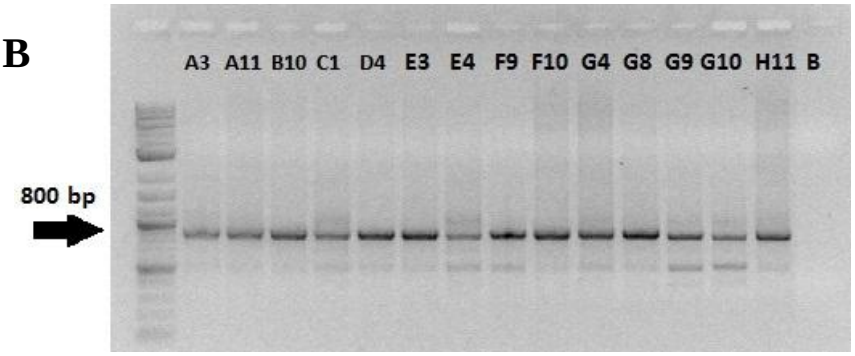



**Fig. 8-A – Screening of CLDN-1 positive phages by cell ELISA on CLDN-1-positive and-negative cells.**
**Fig. 8-B - Analysis by PCR of cDNA encoding scFv fragment (800 bp) from some positive clones**

### *1.3.Production of soluble scFvs*

To prepare the human scFvs as soluble molecules, the pHEN2 or pCANTAB phagemid vector containing the DNA encoding each scFv, was expressed in the bacterial strain SF110. After induction with isopropyl-1-thio-D-galactopyranoside, a periplasmic extract was prepared as described previously (14). Because the DNA encoding the scFv was cloned into the

vector fused to a COOH-terminal hexahistidine sequence, the recombinant scFvs were purified by Immobilized-Metal Affinity Chromatography, as described in Materials and Methods.

To verify whether the soluble anti-CLDN-1 scFvs retained the binding properties of the scFvs displayed on phages, the purified proteins were analyzed by ELISA on the same cell lines previously tested with the anti-CLDN-1 scFvs in their phage format. The results indicated that the anti-CLDN-1 soluble scFvs selectively bind to the antigen bearing cells (data not shown).

### *1.4.Production and purification of anti-CLDN- 1 mAbs*

The cDNAs encoding the scFvs showing optimal binding features have been converted to more stable antibody formats by subcloning the variable regions into two eukaryotic vectors for the expression of H (IgG4 isotype) and L chains and by transfecting the resulting recombinant vectors into EBNA 293 cells, as described in Materials and Methods. The secreted antibodies have been purified by protein A-affinity chromatography and analyzed by SDS-PAGE under reducing conditions (see Fig. 9). The purified antibodies were then tested in cell-based assays for selective binding to cells expressing Claudin-1 (data not shown). Six antibodies (named A2, B9x, D10x, E9C2, F7C2 and H9C1) with the best profile of binding have been selected for further analyses.

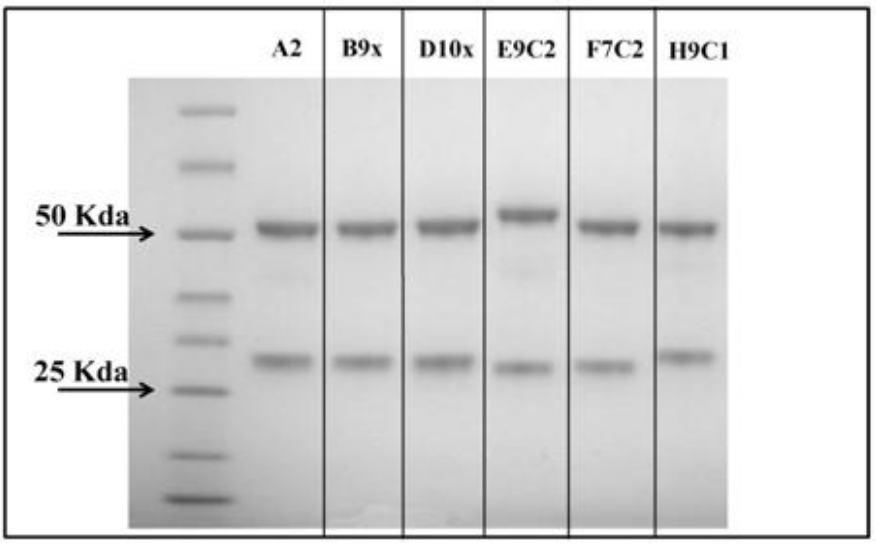



**Fig. 9 - Analysis by SDS-PAGE of purified anti-CLDN-1 mAbs carried out under reducing conditions**

### *1.5.Characterization of anti-CLDN 1 mAbs*

To determine whether the selected antibodies were specific for Claudin-1, Western blotting analyses of HEK t293-CLDN-1 cell lysates previously immunoprecipitated with each mAb were carried out by using a commercial CLDN-1 specific antibody. All the six mAbs recognized a protein of about *M*r 23,000, the molecular weight expected for Claudin-1. In the same experiment a protein corresponding to the same molecular size was recognized by the commercial murine anti-Claudin-1 mAb (Life technology, Paisley, U.K.). No bands were detected when an unrelated mAb was used as a negative control or when untrasfected cells were used for the immunoprecipitation (Fig. 10).

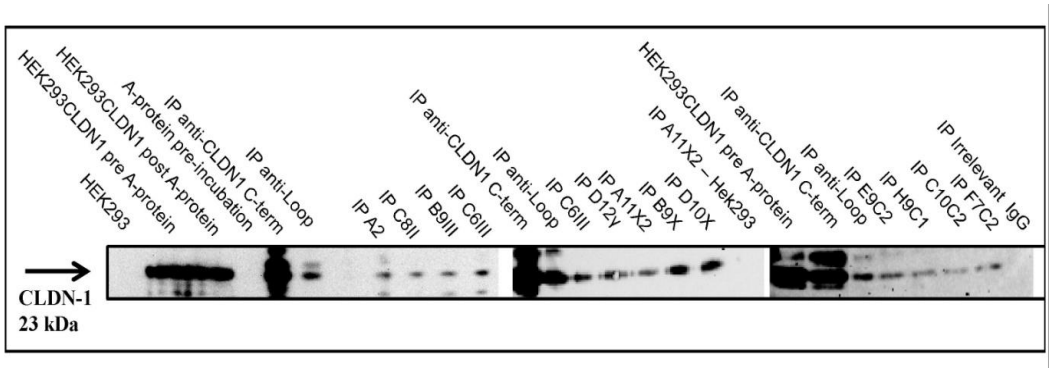



**Fig. 10 - Western blotting analyses with the commercial murine anti-Claudin-1 mAb of HEK t293CLDN-1 cell lysates previously immunoprecipitated with each indicated mAb. As controls were used: lysate from untrasduced HEK t293 cells; lysate from HEK t293CLDN-1 cells before and after incubation with protein-A Sepharose; lysate of HEK t293CLDN-1 cells previously immunoprecipitated with the commercial murine anti-CLDN-1 mAbs (anti-C-term and or-loop); and HEK t293CLDN-1 cell lysate previously immunoprecipitated with an irrelevant IgG**

We then characterized the binding of the anti-CLDN-1 mAbs to Huh7.5, untrasduced Hek t293 or trasduced CLDN-1-HEK t293 cells. As shown in figure 11, all the antibodies bind with high affinity to CLDN-1 positive cells with the only exception of E9C2 mAb which shows a lower affnity. The half-saturating concentrations (corresponding to the apparent Kd) for binding to Claudin-1-positive cells were in the low nanomolar range (Fig. 12) demonstrating that *A2, B9x, D10x, F7C2* and *H9C1* mAbs, selected by phage display, bind with high affinity to CLDN-1 expressed on human

hepatocytes and on CLDN-1-trasduced cells whereas they show poor binding to control untrasduced cells, thus confirming their binding specificity for CLDN-1.

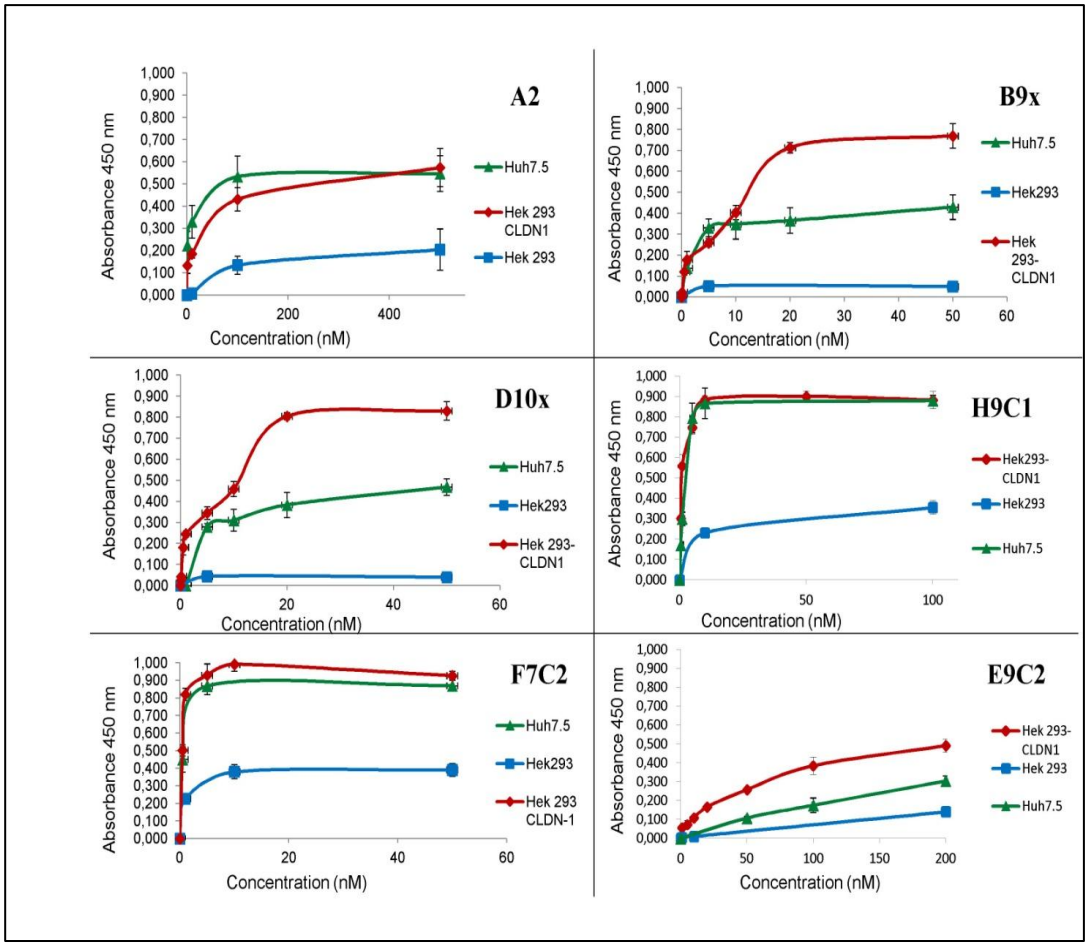
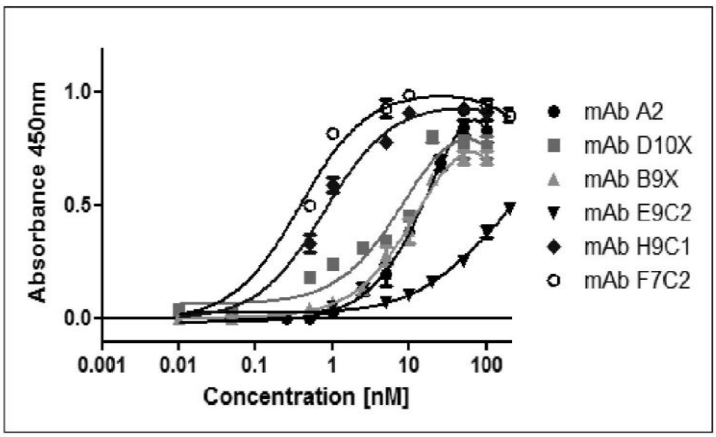



**Fig. 11 - Binding of the 6 anti-CLDN-1 mAbs to Huh7.5 (triangles), untrasduced HEK t293 (squares) and CLDN-1 HEK t293 trasduced cells (rhomboids)**




| mAb | Kd [nM] |
|---|---|
| A2 | 16.0 |
| B9X | 13.1 |
| D10X | 2.9 |
| H9C1 | 0.8 |
| E9C2 | 117 |
| F7C2 | 0.3 |

**Fig. 12 - The half-saturating concentration as measured by the binding curves of the 6 anti-CLDN-1 mAbs to HEK t293-CLDN-1 cell line**

To investigate whether the 6 different anti-CLDN-1 mAbs recognize similar or unrelated epitopes, we performed cross-competition ELISA experiments with the mAbs. Each anti-CLDN-1 mAb was pre-incubated with Huh7.5 cells at saturating concentration and then further incubated for 1 h in the absence or presence of the phages expressing each of the 5 remaining anti-CLDN-1 single chain variable fragments. All of the mAbs were unable to reduce the binding of phages expressing the other scFvs whereas they all strongly reduced the binding of the phages expressing their parental scFv, used as a control (see Fig. 13).

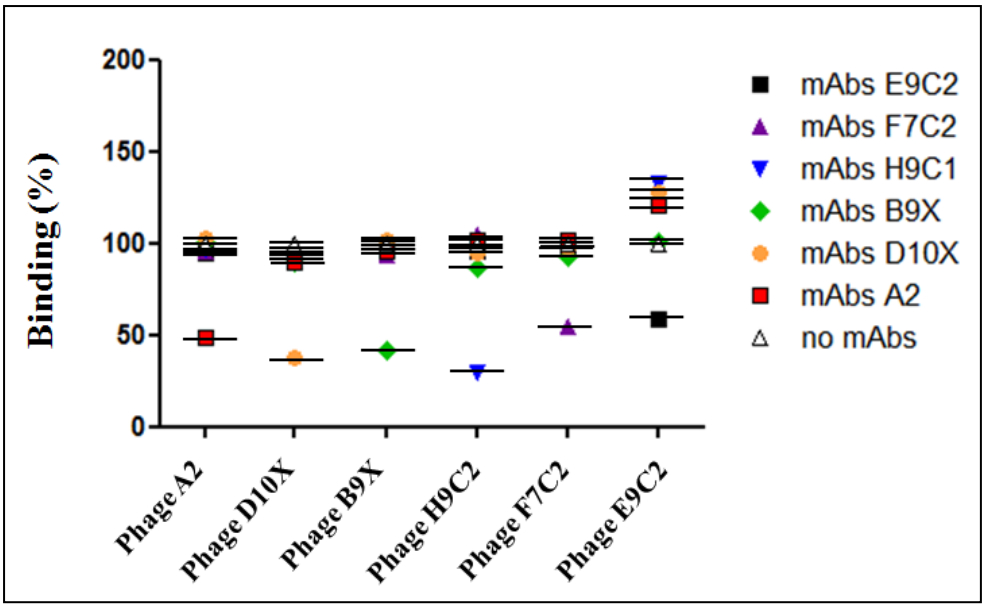



**Fig. 13 – Schematic representation for the competitive binding of the six antibodies. The percentage of binding was calculated as the ratio of the absorbance (450 nm) of each bound detected phage in the absence or in the presence of the indicated antibody**

These results were confirmed by competitive ELISA assays performed by using each biotynilated antibody in the absence or in the presence of the other unlabeled antibodies. The detection of bound antibodies with a streptavidin conjugated secondary antibody confirmed that they do not compete (data not shown).

### *1.6.Neutralization of HCV Infection by human Anti-CLDN-1 Antibodies*

Neutralization assays were performed by using the six different monoclonal antibodies directed against Claudin-1 on four genotype 2 and a chimeric H77/JFH-1 isolates. Virus neutralization was performed at a starting concentration of 100 µg/ml, decreasing 5- fold to 0.032 µg/ml. Neutralization curves are shown in Figure 14. All the mAbs showed neutralization capabilities that varied with isolate as expected. D10x shows an IC50s of less than 20ug/ml against two isolates, JFH-1 and J6 (7.83 ug/ml and 17.26 ug/ml, respectively) and B9X has the lowest IC50 (5.73 ug/ml; JFH-1). These two antibodies were then used in combination, at equal concentrations, to see if

they were antagonistic, synergistic or additive. As shown in Fig. 15, they showed additive effects against all the isolates tested, as expected since they target different epitopes, and thus they could have good clinical value due to minimising escape variants.

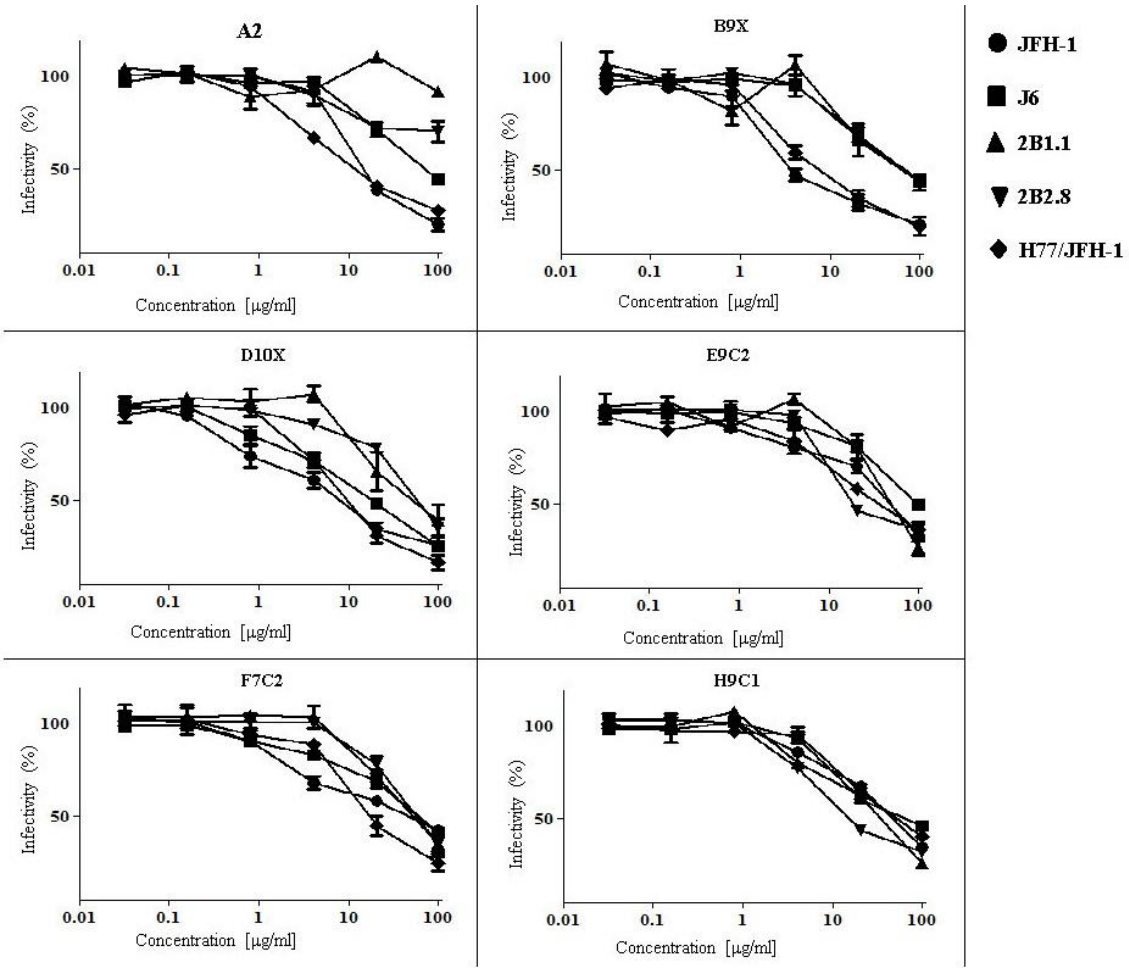



**Fig. 14 - Neutralization curves of six novel anti-claudin antibodies. Neutralization curves against 2B2.8 (inverted triangles), JFH-1 (cyrcles), J6 (squares), 2B1.1 (triangles) and H77/JFH-1 (rhomboids) with six anti-claudin antibodies. All mAbs had neutralization capabilities against the four isolated tested. Antibody preparations were incubated at the indicated concentrations with Huh-7.5 cells for 1 h. Cells were thoroughly washed with PBS and virus was added to the cells. Infection was determined by staining for NS5A after 72 h**

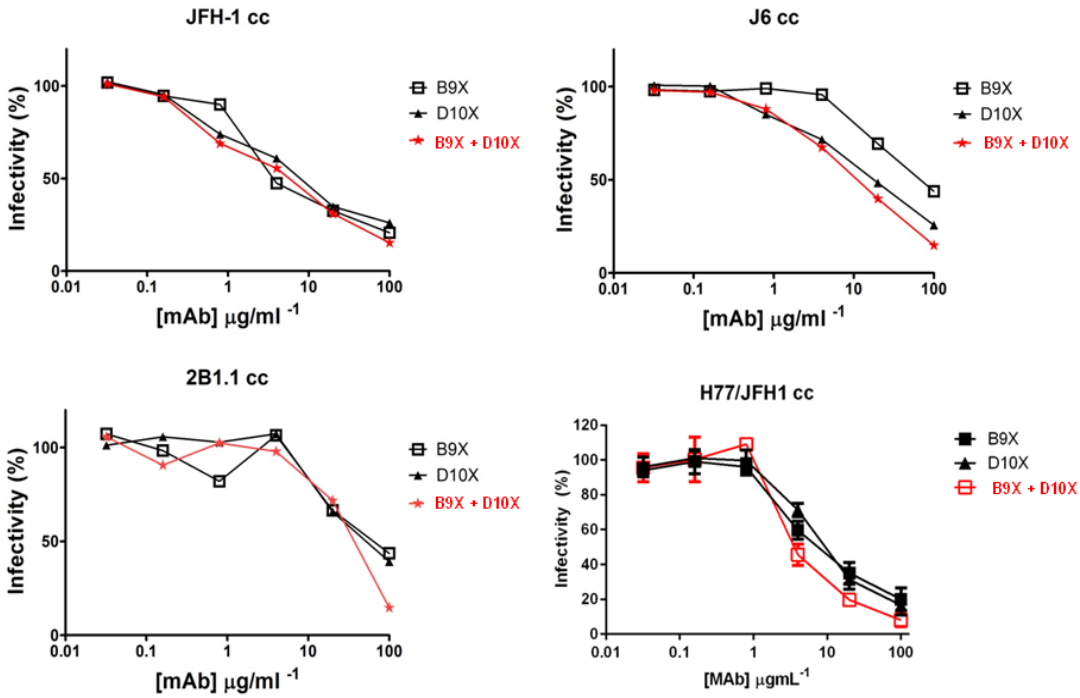



**Fig. 15 - Combinatorial treatment of two different anti-claudin-1 mAbs. Neutralization curves of B9X (squares), D10X (triangles) and the combination of the two antibodies (in Red)**

### *1.7.Side Toxic Effects of Anti-CLDN-1 mAbs*

To address potential toxic effects of anti-CLDN-1 mAbs, we performed cell viability and lysis analyses on treated Huh7.5 cells. Following incubation of hepatoma cells with each of the anti-CLDN-1 antibody for 72 hours at 37°C, tests based on MTT and LDH release were carried out. As shown in Fig. 16 no toxic effects were observed even when very high concentrations (500 nM) were used.

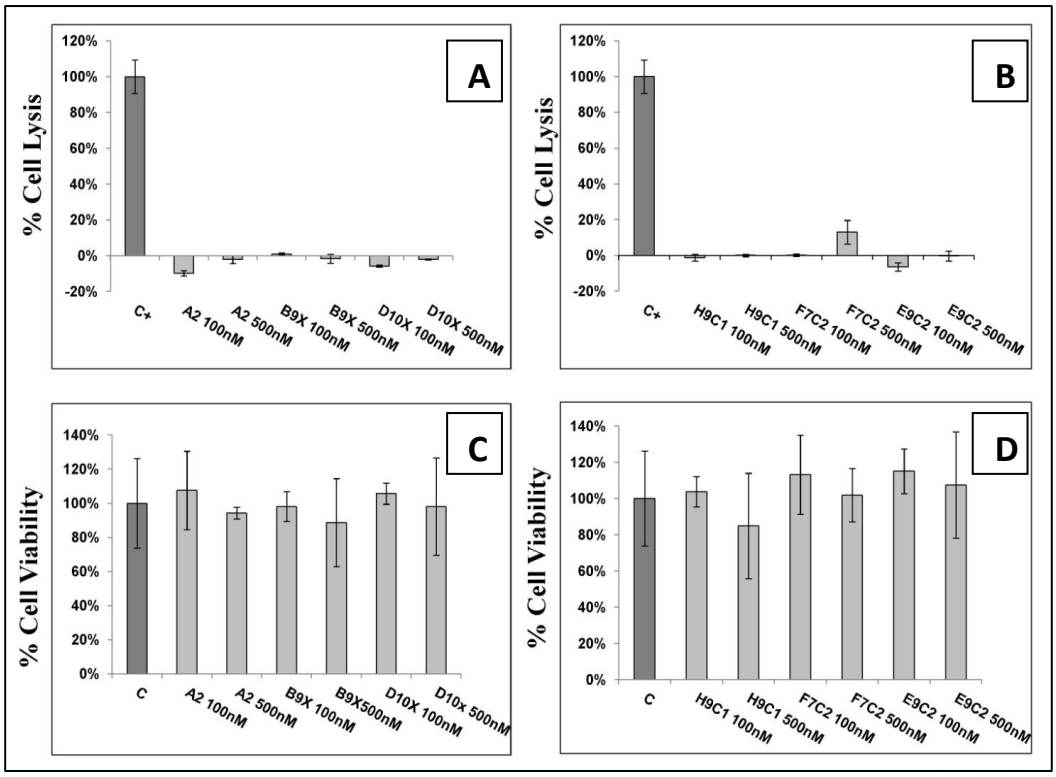



**Fig. 16 - Cytotoxic effects on Huh-7.5 cells of the anti-CLDN-1 mAbs. In panels A and B are shown the percentages of cell lysis after incubation with each indicated anti-CLDN-1 mAb at a concentration of 100 and 500 nM; as positive control, cells were treated with 1% triton X-100. In panels C and D the percentages of cell viability after 72 h of incubation with each indicated anti-CLDN-1 mAb are shown**

## 2. *Human immunoagents for cancer immunotherapy*

### *2.1.Expression and purification of the ImmunoRNase*

The expression and purification of the ImmunoRNase was performed as described in Materials and Methods. The protein eluted by affinity chromatography on Immobilized Metal Ion Affinity Chromatography (IMAC) was analyzed by SDS-PAGE followed by Coomassie staining (Fig. 17-A) and by Western blotting analyses with a mouse HRP-conjugated anti-His monoclonal antibody (Qiagen), as shown in figure 17-B.

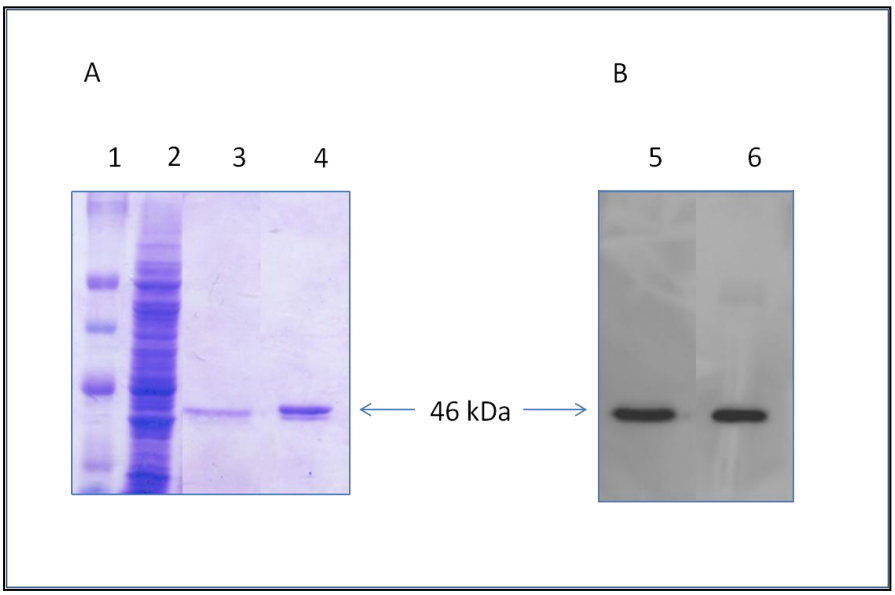



**Fig. 17-A - SDS-PAGE analysis of purified ERB-HP-DDADD-RNase followed by Coomassie staining. *Lane 2*, flow through; *Lane 3*, fractions eluted from the column; *Lane 4*, ERB-HP-RNase, used as a standard molecular weight protein.**
**Fig. 17-B - Western blotting analysis of purified ERB-HP-DDADD-RNase detected with an anti-His antibody. *Lane 5* and *6* as in *Lane 3* and *4*, respectively**

### *2.2.RNase activity and inhibition of ERB-HP-DDADD-RNase*

The RNase activity was tested by the acid-insoluble RNA precipitation assay, as described in Materials and Methods. The engineered immunoRNase (ERB-HP-DDADD-RNase) retains about 40% of the activity of the parental immunoRNase, with a specific activity of 110 U/nmol (Fig. 18). This result is in line with the data previously reported in literature (77) for the mutant (HP-DDADD) and wild type RNases.

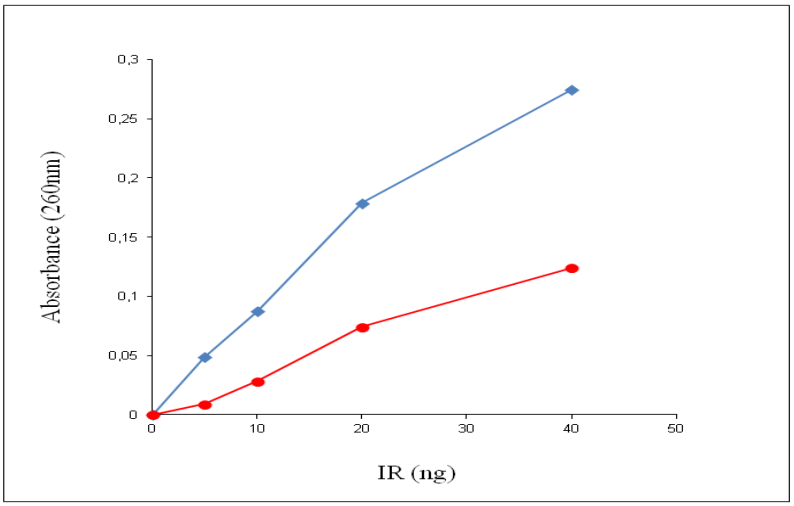



**Fig. 18 - Enzymatic activity of the immunoRNases (IR). Absorbance of soluble degradation products of ERB-HP-RNase (in Blue) or ERB-HP-DDADD-RNase (in Red)**

The sensitivity of the engineered immunoRNase to inhibition by the human inhibitor (RI) was then tested by pre-incubating appropriate amounts of the immunoRNase with increasing concentrations of RI, as described in Materials and Methods. The immunoRNase ERB-HP-DDADD-RNase was found to be not susceptible to inhibition by RI, in contrast to the native immunoRNase ERB-HP-RNase, tested in parallel, which was found to be fully inhibited at RI/IR ratio of about 1 (Fig. 19). The percentage of inhibition was calculated as follows: % inhibition = 100 - (% activity in the presence of RI/activity in the absence of RI).

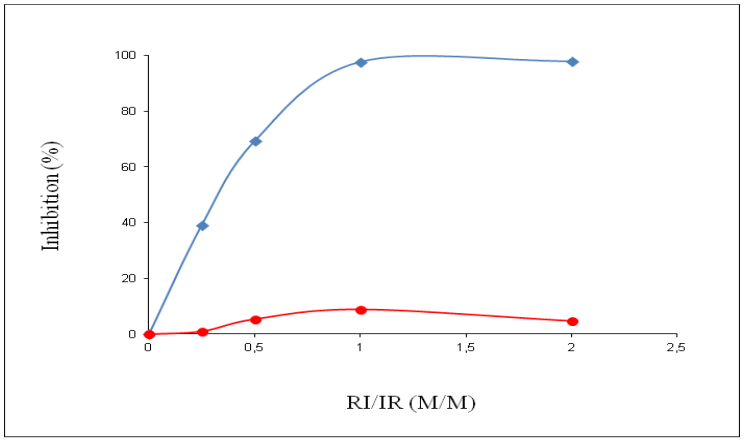



**Fig. 19 - Effects of the RNase inhibitor (RI) on the enzymatic activity of the parental ERB-HP-RNase (in Blue) or the variant ERB-HP-DDADD-RNase (in Red), measured at increasing RI/IR ratios**

### *2.3. Binding of ERB-HP-DDADD-RNase to ErbB2-positive human tumor cells*

The affinity of Erb–HP-DDADD-RNase for the ErbB2 receptor on gastric tumor cells was tested by cell ELISA assays, performed on NCI-N87, MKN7 and AGS cells. In a parallel assay, mammary SKBR3 or JIMT-1 cells, expressing high or moderate levels of ErbB2 receptor, respectively, were used as positive controls, and epidermoid A431 cells, expressing very low levels of ErbB2, as a negative control (data not shown). The results, shown in Fig. 20, indicate that Erb–HPDDADD-RNase retains the specificity of the parental Erb–HP-RNase, binding with a similar high affinity to control SKBR3 and JIMT-1 cells. As for the gastric tumor cells, both immunoRNases bind to MKN7 and NCI-N87 with a high affinity, but do not recognize AGS cells.

To investigate whether the differential binding of the immunoRNases to the three gastric cell lines was due to a differential expression of ErbB2 in these cells, a quantitative analysis of their ErbB2 levels was performed on lysates by western blotting with a commercial anti-ErbB2 mAb. As shown in Fig. 21, ErbB2 is expressed in the tested gastric cell lines NCI-N87 and MKN7 at levels comparable to those found in JIMT-1 breast cancer cells, though lower with respect to those detected in SKBR3 cells. No significant signal corresponding to the receptor was detected in AGS tumor cells, suggesting that these cells express very low levels of ErbB2. These results

indicate a positive correlation between the ErbB2 levels on a cell and the affinity of the immunoRNases for that cell.

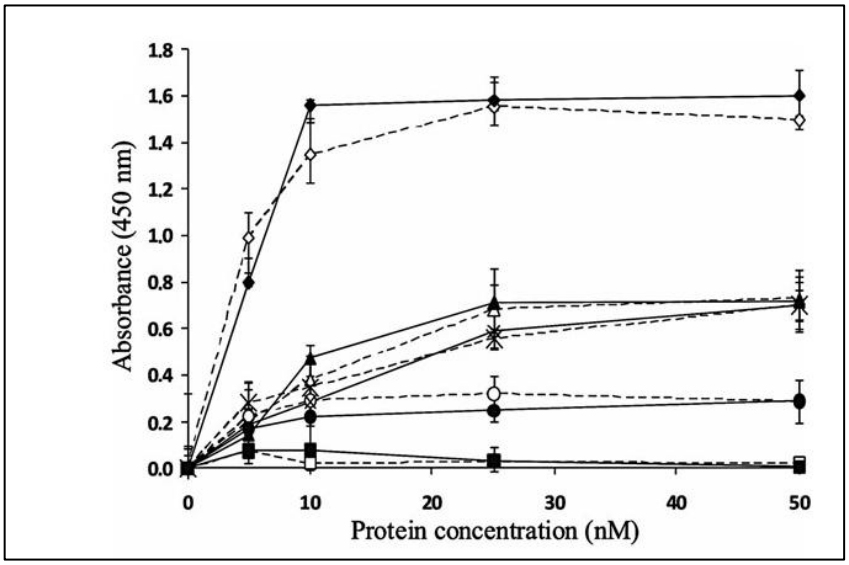



**Fig. 20 - Binding curves of Erb–HP-RNase (dashed lines) or Erb–HP-DDADD-RNase (black lines) to gastric NCI-N87 (triangles), MKN-7 (circles) and AGS (squares) cell lines, as well as to breast SKBR3 (rhomboids) and JIMT-1 (crosses) cell lines, used as positive controls**

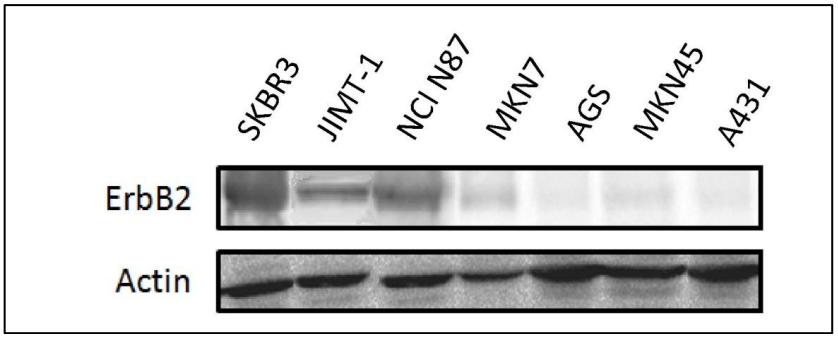



**Fig. 21 - Quantitative analysis by western blotting of ErbB2-receptor levels on gastric (NCI-N87, MKN-7, AGS and MKN-45) cell lines and breast cancer (SKBR3 and JIMT-1) cell lines. The A431 cell line from human epidermoid carcinoma was used as a negative control**

### *2.4. Cytotoxic effects of ERB-HP-DDADD-RNase*

The antitumor effects of the immunoRNase were tested on gastric tumor NCI-N87, MKN-7 and AGS cells in comparison with those observed on other ErbB2-positive tumor cells from different tissues, such as mammary SKBR3 (positive control), JIMT-1 cells resistant to Herceptin and ErbB2-negative epidermoid A431 tumor cells (negative control; data not shown). After a 72 h incubation at 37°C, cell survival was expressed as percentage of viable cells in the presence of an immunoRNase with respect to control cells grown in the absence of the protein. As shown in Fig. 22, Erb–HP-DDADD-RNase was found to inhibit the growth of the gastric tumor cells tested in a dose dependent manner, showing a strong effect on the NCI-N87 cell line, even at low protein concentrations. This immunoRNase is toxic to MKN-7 cells, albeit at a higher protein concentration, and more so than the parental Erb–HP-RNase. Similar results were obtained for mammary JIMT-1 cells, which express ErbB2 levels comparable to those of NCI-N87 and MKN-7 cells, but lower than those of SKBR3 cells, which were found to be equally sensitive to both immunoRNases. No cytotoxic effects were detected on AGS cells, as expected from the low affinity of both immunoRNases to these cells. Indeed, all of the cytotoxicity data indicate a correlation between the level of ErbB2 expression by a tumor cell and its vulnerability to an immunoRNase.

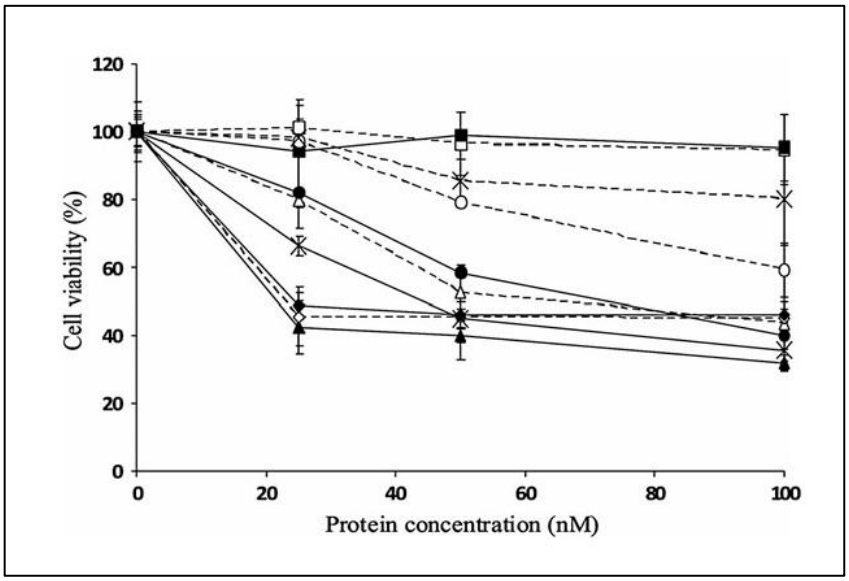



**Fig. 22 - Cytotoxic effects of immunoRNases on tumor cells in vitro. Dose–response curves for gastric NCI-N87 (triangles), MKN-7 (circles) and AGS (squares) cell lines, as well as for breast SKBR3 (rhomboids) and Herceptin-resistant JIMT-1 (crosses) cell lines, treated for 72 h with Erb-HP-RNase (dashed lines) or Erb-HP-DDADD-RNase (black lines)**

### *2.5.In vivo antitumor activity of Erb–HP-DDADD-RNase*

To evaluate the therapeutic effects of Erb–HP-DDADD-RNase, we used ErbB2-positive JIMT-1 cells, clinically resistant to Herceptin, for studies in vivo. As shown in Fig. 23, Erb–HPDDADD-RNase reduces tumor volume substantially, whereas Erb–HP-RNase has a lesser effect. During treatment, the mice did not show signs of wasting or other visible signs of toxicity. Thus, we conclude that Erb–HP-DDADD-RNase exerts antitumor effects stronger than those of the parental immunoRNase, consistent with its resistance to RI.

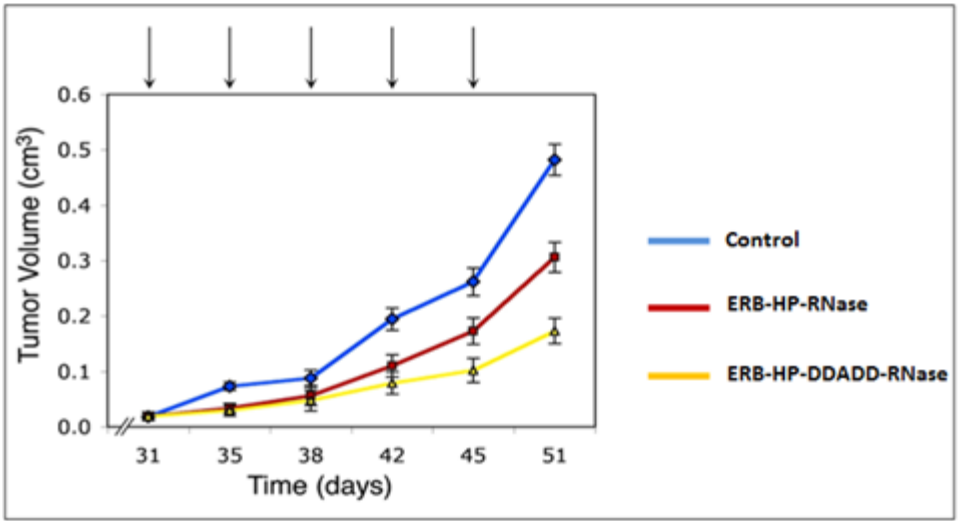



**Fig. 23 - In vivo effects of Erb–HP-DDADD-RNase (in Yellow) or Erb–HP-RNase (in Red) on Herceptin-resistant JIMT-1 tumors induced in mice. Treated mice (n = 5) were injected with Erb–HP-RNase or Erb–HP-DDADDRNase at five doses of 1,2 mg/Kg$^{-1}$ of body weight. Control mice (n = 5) were treated with sterile PBS (in Blue)**

### *2.6.Binding of Erb-hcAb to tumor gastric cells*

To evaluate the therapeutic potential of the compact anti-ErbB2 antibody on the gastric tumor cells mentioned above, ELISA assays on these cells with increasing concentrations of Erb-hcAb or Herceptin, were carried out to test their binding capacity. As shown in Fig. 24, the two antibodies were found to bind to the NCI-N87 and MKN-7 gastric cells tested with high affinities (see Fig. 24), whereas no significant binding to MKN-45 and AGS cells expressing low levels of ErbB2 was detected.

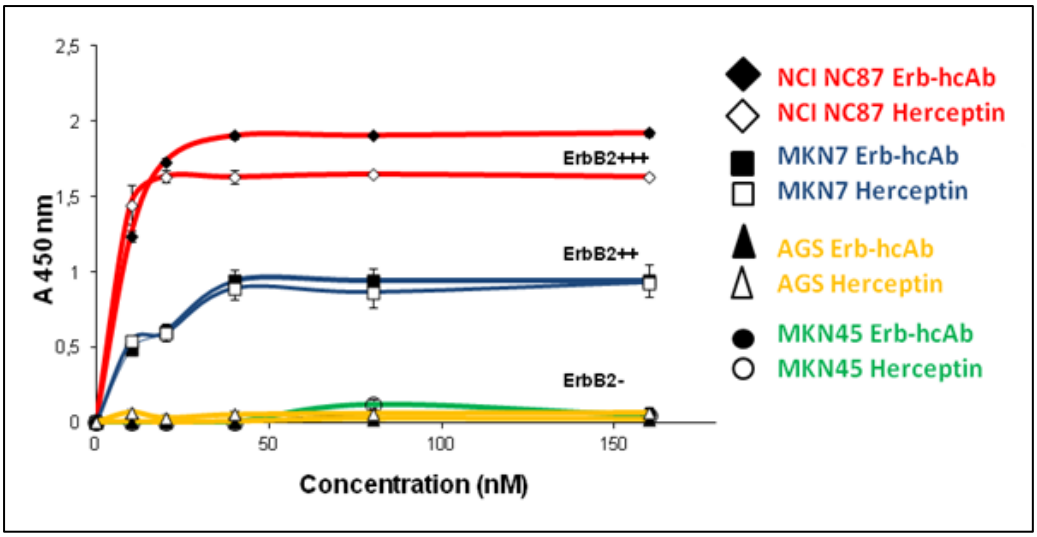



**Fig. 24 - Binding curves of Erb-hcAb (Black symbol) and Herceptin (Empty symbol) to gastric cancer cells: NCI-N87 (in Red), MKN-7 (in Blue), MKN-45 (in Green), or AGS (in Yellow) cells**

### *2.7. In vitro effects of Erb-hcAb on gastric cancer cells*

The antitumor effects of the antibodies were tested on gastric tumor cells by incubating NCI-N87, MKN-7, MKN-45, and AGS cells in the absence or in the presence of increasing concentrations (50–200 nM) of Erb-hcAb or Herceptin. After 72 h, cells were counted and their survival was expressed as percentage of viable cells in the presence of the protein under test, with respect to control cells grown in the absence of the protein. As shown in Fig.25, Erb-hcAb was found to inhibit the growth of ErbB2-positive NCI-N87 and MKN-7 cells tested in a dose-dependent manner, showing a stronger effect with respect to Herceptin when high protein concentrations were tested. In contrast, no significant effects were observed when Erb-hcAb or Herceptin was tested on MKN-45 or AGS cells (Fig. 25) expressing very low levels of ErbB2 (see Fig. 21). These data clearly indicate the specificity of the antiproliferative activity of the anti-ErbB2 antibodies.

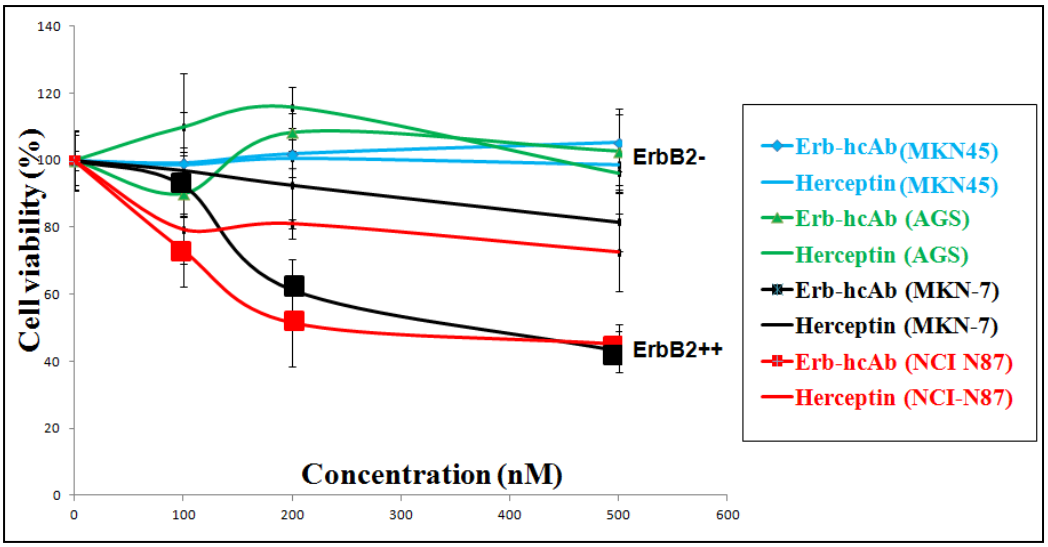



**Fig. 25 - In vitro anti-tumor effects of Erb-hcAb or Herceptin on gastric cancer cells: NCI-N87 (in Red), MKN-7 (in Black), MKN-45 (in Blue), or AGS (in Green) cell lines, treated for 72 h with the indicated antibodies**

### *2.8. Effects of Erb-hcAb on ErbB2 signaling*

We then evaluated by Western blotting analysis the effects of treatment with Erb-hcAb on the expression of proteins involved in the ErbB2 pathway in NCI-N87 cells. Erb-hcAb inhibited the phosphorylation of MAPK and Akt (Fig. 26), thus suggesting that it retains its ability to inhibit the ErbB2 survival pathway in gastric tumor cells.

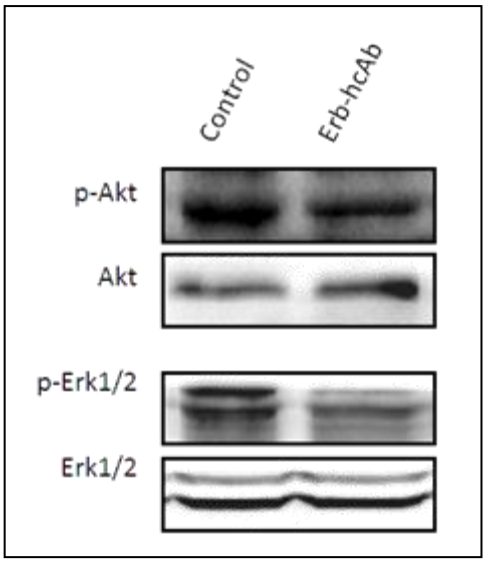



**Fig. 26 - Effects on MAPK and Akt signaling in NCI-N87 tumor gastric cells, as exerted by Erb-hcAb. Reduction in p-Erk1/2 or p-Akt levels was normalized to total Erk2 or Akt, respectively, by phosphor imaging signals detected by Western blotting**

### *2.9.Antibody-dependent cellular cytotoxicity (ADCC) as induced by Erb-hcAb*

To determine the capacity of Erb-hcAb to trigger ADCC toward antigen-expressing NCI-N87 cells, the target cells were incubated for 24 or 48 h with effector PBL in the absence or in the presence of increasing concentrations of Erb-hcAb or Herceptin, used as a positive control. As shown in Fig. 28, Erb-hcAb effectively lysed NCI-N87 target cells in the presence of PBL. The extent of lysis reached almost 35 % of treated cells, whereas Herceptin, used as a positive control, induced about 15–20 % lysis. The basal level of cytotoxicity was measured in the presence of PBL (Fig. 27) or Erb-hcAb alone (data not shown). No effects were detected in parallel assays carried out with ErbB2-negative cells, such as A431 (data not shown), or when Erb-hcAb was replaced by the parental anti-ErbB2 scFv, lacking the Fc domain (data not shown). These results indicate the specificity of the Erb-hcAb-dependent cell-mediated cytolytic activity, clearly based on binding abilities of the immunoagent both to the cognate receptor with its antigen-binding sites and to natural killer cells with its Fc effector domain.

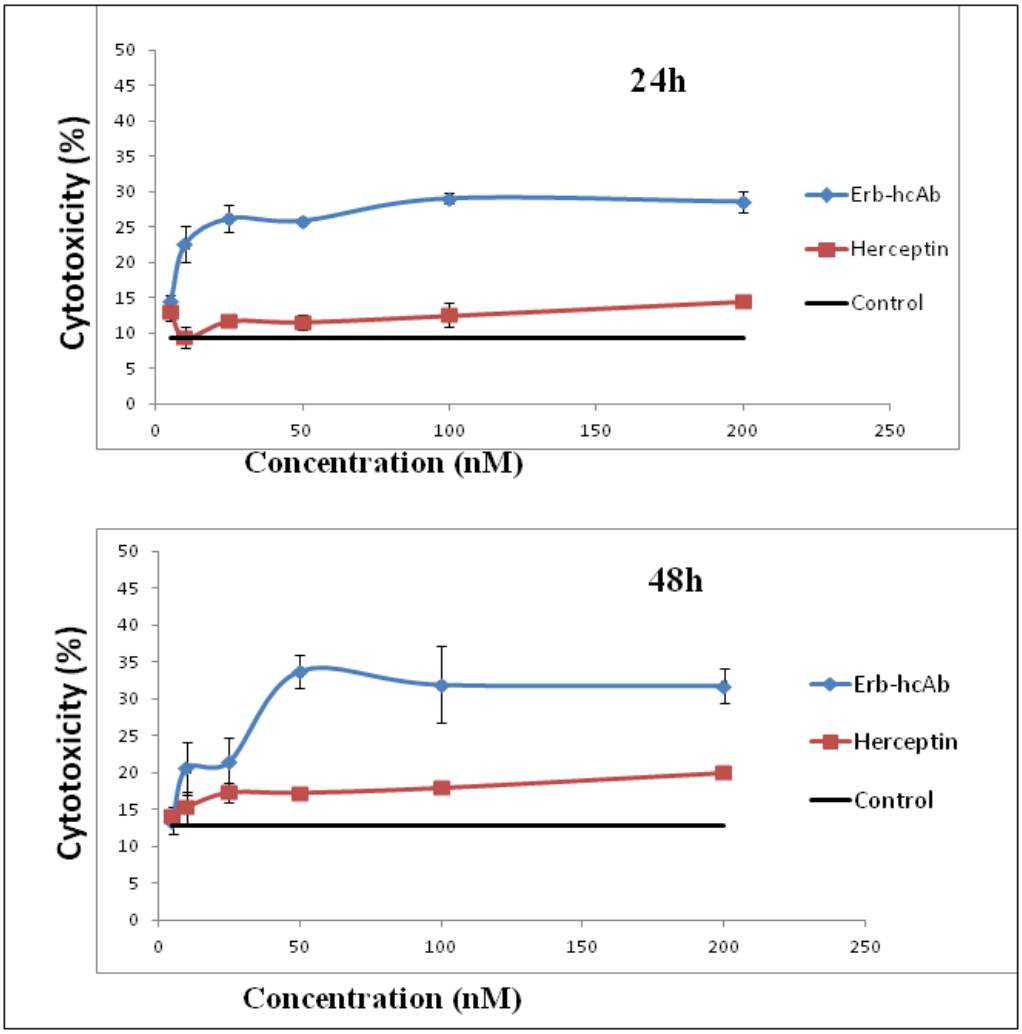



**Fig. 27 - Antibody-dependent cytotoxicity assays on NCI-N87 cells treated for 24 or 48 h with peripheral blood lymphocytes (PBL) as effector cells in the absence (in Black) or in the presence of Erb-hcAb (in Blue) or Herceptin (in Red)**

### *2.10. In vivo antitumor activity of Erb-hcAb*

For in vivo studies, Erb-hcAb was tested on human gastric NCI-N87 cancer cells. The treatment of mice bearing NCI-N87 tumors with seven doses, at 72-h intervals, of 5 $mg/kg^{-1}$ body wt of Erb-hcAb induced a significant reduction in tumor volume (Fig. 28). In parallel experiments, we

tested the effects of trastuzumab, administered as already indicated for Erb-hcAb. Figure 28 shows that trastuzumab, in contrast to Erb-hcAb, showed only slight effects on NCI-N87 gastric tumors. No growth inhibition was observed in mice treated with PBS. During the period of treatment, the animals did not show signs of wasting or other visible signs of toxicity.

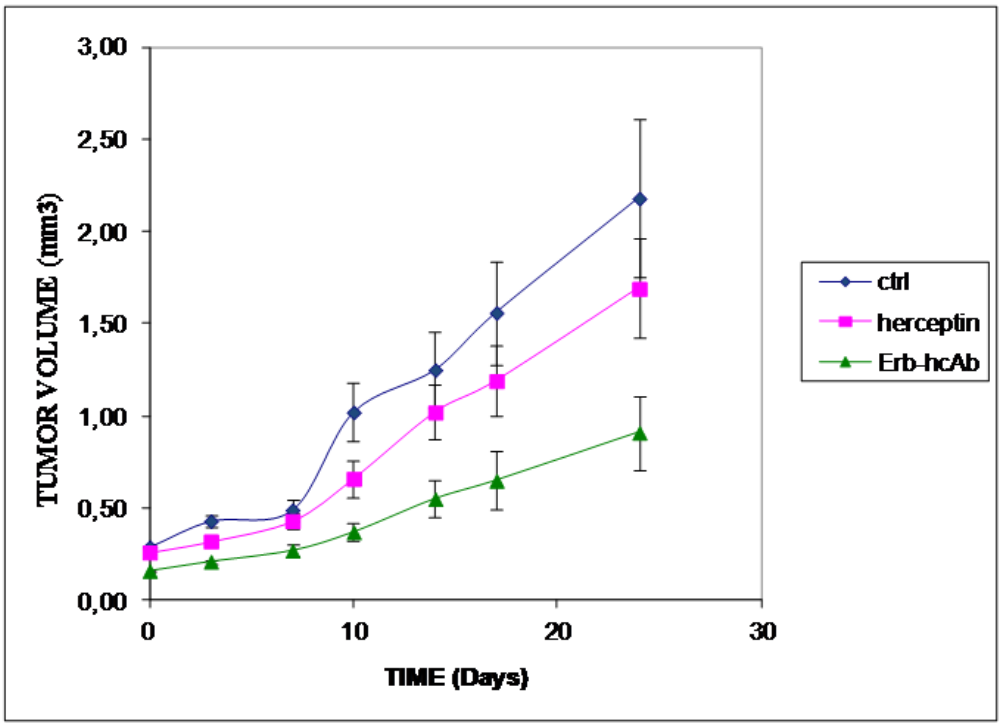



**Fig. 28 - In vivo antitumor effects of Erb-hcAb on gastric xenografts. Tumor growth was measured in mice inoculated subcutaneously with NCI-N87 cells from a human gastric tumor. Treated mice (triangles) were injected with Erb-hcAb at doses of 5 mg/kg body weight. Injections were repeated seven times at 72-h intervals. Control mice were treated with sterile PBS solution (rhomboids), or with equimolar doses of trastuzumab (squares)**

# DISCUSSION

## *1. Human antiviral immunoagents*

Hepatitis C virus (HCV), the prototypical member of the Hepacivirus genus within the Flaviviridae family, is a blood-borne pathogen that affects approximately 3% of the global population. Approximately 60%–80% of infections lead to hepatitis C, a chronic degenerative disease of the liver, which can progress over time to liver fibrosis, steatosis, cirrhosis and hepatocellular carcinoma (HCC). HCV is associated with the majority of newly diagnosed hepatocellular carcinomas in the United States (78, 79) and is currently the leading cause of liver transplants worldwide (19).

An HCV vaccine is not available and current therapies based on pegylated interferon-alpha and ribavirin are poorly effective, costly, toxic and have limited efficacy depending upon the infecting genotype. Telaprevir and Boceprevir are the drugs targeting the virus protease, in clinical use since 2011, that show side toxic effects. Furthermore, a rapid appearance of drug-resistant viruses emerge during treatment (20 - 24), highlighting the need for combination therapies with agents targeting diverse viral and host cell pathways.

Monoclonal antibodies are particularly attractive because they are a validated class of therapeutics with proven efficacy, safety in a number of clinical applications, and an established production process. Furthermore, with the advent of humanized or fully human mAbs, there are no risks of immune reaction against the antibody (80 – 83). Overall, human mAbs have superior safety and pharmacokinetic profiles than the current antivirals and emerging DAAs particularly in the highly susceptible population of HCV positive liver transplanted patients.

Anti-viral strategies based upon biologics have been successfully established for other viruses. Clinical proof of concept has been obtained for the HIV fusion inhibitor Fuzeon (Enfuvirtive), a synthetic peptide that blocks HIV entry into target cells (84), and for a monoclonal antibody against the HIV CCR5 co-receptor (PRO-140) (85). Synagis (Palivizumab), a humanized monoclonal antibody against the fusion protein of the respiratory syncytial virus (RSV), is the current standard of care for the prevention of RSV infections in infants that are at high-risk because of prematurity or other medical problems such as congenital heart disease (86). In the case of

Hepatitis B virus (HBV) chronic patients undergoing liver transplant, the use of hepatitis B immune globulin (HBIG) after liver transplantation, concurrent with NUCs at the time of, or immediately after, liver transplantation, is considered to be the standard of care in order to prevent reinfection of the graft.

A similar strategy could be adopted for the prevention of liver graft re-infection in HCV LT patients. However, HCV displays a very high inter- and intra-individual genetic variability especially in the E1 and E2 envelope glycoproteins where neutralizing determinants are present. Therefore antiviral neutralizing antibodies are effective at best only against some viral variants (15). Accordingly, passive immunotherapy in LT patients with polyclonal or monoclonal antibodies against the viral envelope can only transiently lower RNA levels, but do not prevent re-infection presumably due to the large heterogeneity in the infecting viral population (25, 26). In addition, it was shown that HDL can reduce the neutralizing effect of anti-HCV antibodies (27, 28), raising additional concerns about the efficacy of anti-HCV antibodies for active or passive immunotherapy.

For the above mentioned reasons, the recent discovery of cell surface molecules required for HCV viral entry may offer a radically novel perspective for the development of new preventative and therapeutic agents. As an alternative to the development of anti-HCV antibodies, the development of anti-receptor antibodies are less likely to be prone to sequence changes in viral proteins.

The human scavenger receptor class B type I (SRB1) is an essential receptor for HCV (29, 31, 87, 88). It has been shown that SRB1 is required for infection by different HCV subtypes (30, 88) supporting the hypothesis that the same SRB1 protein element is responsible for the recognition of different HCV E2 glycoproteins despite their high level of sequence variability. Furthermore, monoclonal antibodies (mAb) against native human SRBI block HCV virus infection of Huh7.5 hepatoma cells in a dose-dependent manner (31). Most importantly, the neutralization potency of these mAbs was not affected by HDL (31), suggesting that an anti-HCV immunotherapy based on human antibodies against SRB1 may overcome the problem of desensitization of HCV neutralization by antibodies against the virus.

An alternative target for the anti-HCV immunotherapy is the tight junction protein CLDN-1, essential for the late step of virus entry, spread and persistence of HCV infection (9). Recently, rat monoclonal antibodies against CLDN1 have been produced by using genetic immunization. These anti-CLDN-1 mAbs have been found to be effective for inhibition of all major genotypes as well as highly variable HCV quasispecies isolated from individual patients, blocking cell entry of highly infectious escape variants of

HCV that were resistant to neutralizing antibodies (34). However, a full success of present-day immunotherapy could be still hindered by immune response against non-human, or even humanized antibodies.

Thus, to generate human monoclonal antibodies against CLDN-1 we selected single chain (scFv) libraries displayed on filamentous bacteriophage M13 by using a subtractive selection strategy based on the use of several different combinations of CLDN-1 -positive and -negative cell lines devised to guarantee the selection of a large number of clones with high specificity for CLDN-1. Phage binders showing the highest degree of specificity for CLDN-1 were converted into whole human IgG4. The IgG4 isotype was chosen because it lacks ADCC or ability to mediate NK and complement activity. Furthermore, IgG4 have longer half-life.

Six human anti-CLDN-1 mAbs, named A2, B9x, D10x, E9C2, F7C2 and H9C1, were further characterized and found capable to selectively bind to CLDN1-positive cells with a variety of different affinities ranging from about 100 down to 0.3 nM.

All the antibodies were capable to potently inhibit cell entry of different HCV isolates in a dose-dependent manner. However surprisingly there was no correlation between their binding affinities and their neutralization efficacy as the best binder F7C2 (Kd=0.3 nM) showed similar inhibitory effects (EC50= 20-60 μg/mL) when compared to those of E9C2 (Kd=117 nM), and the two most potent antibodies were found to be B9X and D10X with an EC 50 down to 6 μg/mL even though their affinities are not the highest, being 13.1 and 2.9 nM, respectively.

This finding can be explained by considering that these antibodies recognize different epitopes of CLDN1 and thus could have different mechanisms of action, thus allowing for combinatorial treatment. Indeed an increased antiviral potency has been observed when the two antibodies B9x and D10x, were used in combination, thus suggesting that their effects are at least additive.

Interestingly, the doses of these human anti-CLDN1 mAbs required for efficient inhibition (EC50 of 5–50 μg/mL) were similar to those observed for the six rat anti-CLDN-1 antibodies mentioned above, that on the contrary cannot be used in combinatorial treatment as they all recognize a similar epitope.

It is noteworthy that these concentrations are lower than those used in clinic for antibody anti-HBs (hepatitis B virus surface antigen) required for prevention of HBV infection (100-500 μg/ml) after liver transplantation (89), and in a similar range of approved therapeutic mAbs used in cancer or antiviral treatment (90, 91), allowing for the possibility to use these antibodies in clinical therapy.

On the basis of these results we can conclude that these novel fully human anti-CLDN-1 antibodies may provide new anti-HCV therapeutic opportunities either in monotherapy or in combination with other antibodies targeting different HCV receptors or other novel anti-viral drugs to increase the number of responders and to prevent re-infection after liver trasplantation.

## *2. Human immunoagents for cancer therapy*

Immunotherapy is a valid alternative strategy in anti-cancer therapy, useful to overcome the limits of the conventional therapeutic approaches, currently used, such as chemotherapy and radiotherapy. The ErbB2 receptor, highly expressed on many tumor cells of different origin, especially in human breast cancer, is an attractive tumor target.

The first ErbB2-targeted therapeutic immunoagent approved by FDA is Herceptin, a humanized monoclonal antibody widely used for immunotherapy of ErbB2-positive breast cancer. However it can engender cardiotoxicity, and a high fraction of breast cancer patients is resistant to Herceptin treatment. For these reasons, novel anti-ErbB2 immunoagents, such as Erb-hcAb and ERB-HP-DDADD-ImmunoRNase, have been generated in our laboratory by using a human scFv selected by phage display technology. These novel immunoagents were found to bind selectively to tumor cells expressing ErbB2 and to inhibit their growth in vitro and in vivo, with no effects on ErbB2-negative cells. In this study we investigated their therapeutic effects on gastric tumor cells and on breast cancer cells less sensitive or fully resistant to Herceptin treatment, respectively.

We demonstrate that Erb-hcAb is effective on gastric cancer cell lines with ErbB2 overexpression, showing an antitumor effect more potent than that observed for Herceptin. These differences could be explained by the recent interesting finding that Erb-hcAb recognizes an epitope different from that recognized by Herceptin (48, 92). Moreover, our data show that Erb-hcAb induces both ADCC and apoptosis of these cells (93).

Similar antitumor efficacy has been for ERB-HP-DDADD-RNase, a novel fully human immunoRNase targeting the ErbB2 receptor. This immuno-conjugate, made up of Erbicin and a RNase variant resistant to the inhibitor, retains about 40% of the enzymatic activity of the parental immunoRNase but, at difference with the latter, it is fully resistant to RI inhibition. Indeed, ERB-HP-DDADD-RNase inhibits selectively and efficiently the growth of Herceptin-resistant breast and gastric cancer cells *in*

*vitro* and *in vivo* with a more potent cytotoxic action with respect to that of the parental ERB-HP-RNase. This different toxicity is highlighted when the proteins are tested on cell lines with a lower expression of ErbB2 receptor, in which a less efficient internalization of the immunoRNases in the cytosolic compartment does not affect the cytotoxicity of the engineered RI-resistant ERB-HP-DDADD-RNase, whereas it leads to fully inhibition of the parental ERB-HP-RNase.

In conclusion, these results indicate that both Erb-hcAb and ERB-HP-DDADD-RNase confirm their potential therapeutic effect, proving that they could be useful for the treatment of different types of carcinomas less sensitive or resistant to Herceptin treatment.

# REFERENCES


1 - Hossein Keyvani, Mehdi Fazlalipour, Seyed Hamid Reza Monavari, Hamid Reza Mollaie. "Hepatitis C Virus - Proteins, Diagnosis, Treatment and New Approaches for Vaccine Development". *Asian Pacific Journal of Cancer Prevention*, 13, 5917-35 (2012).

2 - De Vicente J., Hendricks R.T., Smith D.B., et al. "Non-nucleoside inhibitors of HCV polymerase NS5B. Part 2: synthesis and structure–activity relationships of benzothiazine-substituted quinolinediones". Bioorganic and amp. *Med. Chemistry Letters*, 19, 3642-6 (2009).

3 - Chatel-Chaix L., Melancon P., Racine M.E., et al. "Y-box-binding protein 1 interacts with hepatitis C virus NS3/4A and influences the equilibrium between viral RNA replication and infectious particle production". *J. Virol.*, 85, 11022-37 (2011).

4 - Burlone M.E., Budkowska A. "Hepatitis C virus cell entry: role of lipoproteins and cellular receptors", *J. Gen. Virol.*, 90, 1055-70 (2009).

5 - Furuse M., Fujita K., et al. "Claudin-1 and -2: novel integral membrane proteins localizing at tight junction with no sequence similiarity to Occludin". *Journal of Cell Biology*, 141, 1539-1550 (1998).

6 - Colegio O.R., Van Itallie C.M., Mccrea H.J., Rahner C., Anderson J.M. "Claudins create charge-selective channels in the paracellular pathway between epithelial cells". *Am. J. Physiol. Cell Physiol.*, 283, 142–47 (2002).

7 - Fujita K., Katahira J., Horiguchi Y., Sonoda N., Furuse M., Tsukita S. "Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein". *FEBS Lett.*, 476, 258–61 (2000).

8 - Itoh M., Furuse M., Morita K., Kubota K., Saitou M., Tsukita S. "Direct binding of three tight junction–associated MAGUKs, ZO-1, ZO-2 and ZO-3, with the COOH termini of claudins". *J. Cell Biol.*, 147, 1351–63 (1999).

9 - Matthew J., Evans, Thomas von Hahn. "Claudin-1 is a hepatitis C virus co-receptor requied for a late step in entry". *Nature*, 446, 10.1038 (2007).

10 - Liu S., Yang W., Shen L. et al. "Tight junction proteins Claudin-1 and Occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection". *J. Virol.*, 83, 2011-2014 (2009).

11 - Yang W., Qiu C., Biswas N. et al. "Correlation of the tight junction-like distribution of Claudin-1 to the cellular tropism of hepatitis C virus". *J. Biol. Chem.*, 283, 8643-8653 (2008).

12 - Timpe J.M., Stamataki Z., Jennings A., Hu K., Farquhar M.J., et al. "Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies". *Hepatology*, 47, 17–24 (2008).

13 - Brimacombe C.L., Grove J., Meredith L.W., Hu K., Syder A.J., et al. "Neutralizing antibody-resistant hepatitis C virus cell-to-cell transmission". *J Virol.*, 85, 596–605 (2011).

14 - Wedemeyer H., Dore G. J., Ward J. W. "Estimates on HCV disease burden worldwide". *Journal of viral Hepatitis*, 22, 1-5 (2015).

15 - Dustin L.B., Rice C.M. "Flying under the radar: the immunobiology of hepatitis C". *Annu Rev Immunol.*, 25, 71-99 (2007).

16 - Davis G.L. et al. "Projecting future complications of chronic hepatitis C in the United States". *Liver Transpl.*, 9, 331–338 (2003).

17 - Neumann A.U. et al. "Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy". *Science*, 282, 103-7 (1998).

18 - Manns M.P. et al. “The way forward in HCV treatment finding the right path”. *Nat. Rev. Drug Discov*., 12, 991-1000 (2007).
19 - Brown R.S. et al. “Hepatitis C and liver transplantation”. *Nature*, 436, 973-978 (2005).
20 - Welsch C. et al. “Molecular basis of telaprevir resistance due to V36 and T54 mutations in the NS3-4A protease of the hepatitis C virus”. *Genome Biol*., 9 (1), R16 (2008).
21 - Dabbouseh N.M., Jensen D.M. “Future therapies for chronic hepatitis C”. *Nat. Rev., Gastroenterol. Hepatol*., 10, 268–76 (2013).
22 - Liang T.J., Ghany M.G. “Current and future therapies for hepatitis C virus infection”. *N. Engl. J. Med*., 368, 1907–17 (2013).
23 - Chung R.T., Baumert T.F. “Curing chronic hepatitis C, the arc of a medical triumph”. *N. Engl. J. Med*., 370, 1576–8 (2014).
24 - Pawlotsky J.M. “Treatment failure and resistance with directacting antiviral drugs against hepatitis C virus”. *Hepatology*, 53, 1742-1751 (2011).
25 - Davis G.L. et al. “A randomized, open-label study to evaluate the safety and pharmacokinetics of human hepatitis C immune globulin (Civacir) in liver transplant recipients”. *Liver Transpl*., 11, 941–949 (2005).
26 - Schiano T.D. et al. “Monoclonal antibody HCV-AbXTL68 in patients undergoing liver transplantation for HCV: results of a phase 2 randomized study”. *Liver Transpl*., 12 (9), 1381-9 (2006).
27 - Dreux M. et al. “High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI”. *J. Biol. Chem*., 281, 18285-95 (2006).
28 - Voisset C. et al. “High-density lipoproteins reduce the neutralizing effect of hepatitis C virus (HCV)-infected patient antibodies by promoting HCV entry”. *J. Gen. Virol*., 87, 2577-81 (2006).
29 - Scarselli E. et al. “The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus”. *EMBO J*., 21, 5017-5025 (2002).
30 - Bartosch B. et al. “Cell entry of hepatitis C virus requires a set of coreceptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor”. *J. Biol. Chem*., 278, 41624-30 (2003).
31 - Catanese M.T. et al. “A High-avidity monoclonal antibodies against the human scavenger class B type I receptor efficiently block hepatitis C virus infection in the presence of high-density lipoprotein”. *J. Virol*., 81, 8063-71 (2007).
32 - Evans. “Claudin-1 is a hepatitis C virus co-receptor required a late step in entry”. *Nature Letters*, 446, 801-806 (2007).
33 - Ploss A., Evans M.J., Gaysinskaya V.A., Panis M., You H., De Jong Y.P., Rice C.M. “Human occludin is a hepatitis C virus entry factor required for infection of mouse cells”. *Nature*, 457, 882-6 (2009).
34 - Fofana I., Krieger S.E., Grunert F., Glauben S., Xiao F., Fafi-Kremer S., Soulier E., Royer C., Thumann C., Mee C.J., McKeating J.A., Dragic T., Pessaux P., Stoll-Keller F., Schuster C., Thompson J., Baumert T.F. “Monoclonal anti-Claudin-1 antibodies prevent hepatitis C virus infection of primary human hepatocytes”. *Gastroenterology*, 139, 953-964 (2010).
35 - Watters J. M., Telleman P. and Junghans R. P. “An optimized method for cell-based phage display panning”. *Immunotechnology*, 3, 21–29 (1997).
36 - World Cancer Report-World Health Organization. Edited by Bernard W. Stuart and Paul Kleiheus (2006).
37 - Slamon D.J. et al. “Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer”. *Science*, 244, 707-712 (1989).

38 - Tagliabue E. et al. "Selection of monoclonal antibodies which induce internalization and phosphorylation of p185HER2 and growth inhibition of cells with HER2/NEU gene amplification". *Int. J. Cancer*, 47, 933-937 (1991).

39 - Fukushige S. et al. "Localization of a novel v-erbB-related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line". *Mol. Cell. Biol.*, 6, 955-958 (1986).

40 - Stebbing J. et al. "Herceptin (trastuzumab) in advanced breast cancer". *Cancer Treat. Rev.*, 26, 287-290 (2000).

41 - Slamon D.J. et al. "Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2". *N. Engl. J. Med*., 344, 783-792 (2001).

42 - Natha R. et al. "Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer". *Nat. Clin. Pract. Oncol*., 3, 269-80 (2006).

43 - Seidman A. et al. "Cardiac dysfunction in the trastuzumab clinical trials experience". *J. Clin. Oncol*., 20, 1215-1221 (2002).

44 - Sparano J.A. "Cardiac toxicity of trastuzumab (Herceptin): implications for the design of adjuvant trials". Semin. Oncol., 28, 20–27 (2001).

45 - Burris H.A., Hurwitz H.I., Dees E.C. et al. "Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas". *J. Clin. Oncol*., 23, 5305–5313 (2005).

46 - Perez E.A., Suman V.J., Davidson N.E. et al. "HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 intergroup adjuvant trial". *J. Clin. Oncol*., 24, 3032–3038 (2006).

47 - Fedele C., Riccio G., Malara A.E., D'Alessio G., De Lorenzo C. "Mechanisms of cardiotoxicity associated with ErbB2 inhibitors". *Breast Cancer Res Treat*., 134 (2), 595–602 (2012).

48 - Troise F., Monti M., Merlino A. et al. "A novel ErbB2 epitope targeted by human antitumor immunoagents". *FEBS J*., 278, 1156–1166 (2011).

49 - De Lorenzo C., Palmer D.B., Piccoli R., Ritter M.A., D'Alessio G. "A new human antitumor immunoreagent specific for ErbB2". *Clin. Cancer Res*., 8, 1710-1719 (2002).

50 - De Lorenzo C., Tedesco A., Terrazzano G., Cozzolino R., Laccetti P., Piccoli R. et al. "A human, compact, fully functional anti-ErbB2 antibody as a novel antitumour agent". *Br. J. Cancer*, 91 (6), 1200–4 (2004).

51 - De Lorenzo C., D'Alessio G. "Human anti-ErbB2 immunoagents: immunoRNases and compact antibodies". *FEBS J*., 276 (6), 1527–35 (2009).

52 - Chien K.R. et al. "Myocyte survival pathways and cardiomyopathy: implications for trastuzumab cardiotoxicity". Semin. Oncol., 27 (6 suppl 11):9–14 (discussion 92–100) (2000).

53 - Fedele C., Riccio G., Coppola C., Barbieri A., Monti M.G., Arra C., et al. "Comparison of preclinical cardiotoxic effects of different ErbB2 inhibitors". *Breast Cancer Res. Treat*., 133 (2), 511–21 (2012).

54 - Riccio G., Esposito G., Leoncini E., Contu R., Condorelli G., Chiariello M., et al. "Cardiotoxic effects, or lack thereof, of anti-ErbB2 immunoagents". *FASEB J*,. 23 (9), 3171–8 (2009).

55 - Gelardi T., Damiano V., Rosa R., Bianco R., Cozzolino R., Tortora G. et al. "Two novel human anti-ErbB2 immunoagents are active on trastuzumab-resistant tumours". *Br. J. Cancer*, 102(3), 513–9 (2010).

56 - Pastan I. et al. "Recombinant toxins for cancer treatment". *Science*, 254, 1173-1177 (1991).
57 - Carter P. et al. "Improving the efficacy of antibody-based cancer therapies". *Nature Reviews Cancer*, 1, 118-129 (2001).
58 - Zhang Q. et al. "Monoclonal antibodies as therapeutic agents in oncology and antibody gene therapy". *Cell Research*, 2, 89-99 (2007).
59 - Weiner L.M. et al. "Phase I evaluation of an anti-breast carcinoma monoclonal antibody 260F9-recombinant ricin A immunoconjugate". *Cancer Res*., 49, 4062-4067 (1989).
60 - Schindler J. et al. "The toxicity of deglycosylated ricin A chain-containing immunotoxins in patients whit non-Hodgkin's lymphoma is exacerbated by prior radiotherapy: a retrospective analysis of patients in five clinical trials". *Curr. Opin. Oncol*., 13, 168-175 (2001).
61 - Rybak S.M. et al. "Natural and engineered cytotoxic ribonucleases: therapeutic potential". *Exp. Cell. Res*., 253, 325-335 (1999).
62 - De Lorenzo C. et al. "From immunotoxins to immunoRNase". *Curr. Pharm. Biotechnol*., 9, 210-214 (2008).
63 - De Lorenzo C. et al. "A fully human antitumor immunoRNase selective for ErbB2-positive carcinomas". *Cancer Res*., 64, 4870-4874 (2004).
64 - Haigis M.C. et al. "Ribonuclease inhibitor as an intracellular sentry". *Nucl. Acids Res*., 31, 1024-1032 (2003).
65 - Dickson K.A. et al. "Ribonuclease inhibitor: structure and function". *Prog. Nucl. Acid Res. Mol. Biol*,. 80, 349-374 (2005).
66 - Kobe B. et al. "A structural basis of the interactions between leucin-rich repeats and protein ligands". *Nature*, 374, 183-186 (1995).
67 - Johnson J. et al. "Inhibition of human pancreatic ribonuclease by the human ribonuclease inhibitor protein". *J. Mol. Biol*., 368, 434-449 (2007).
68 - Riccio G., D'Avino C., Raines R. T., De Lorenzo C. "A novel fully human antitumor ImmunoRNase resistant to the RNase inhibitor". *Protein Engineering, Design, and Selection*, 26 (3), 243-8 (2013).
69 - Sambrook J., Fritsch E.F. and Maniatis T. "Molecular Cloning: A Laboratory Manual", Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 9.16 (1989).
70 - De Lorenzo C., Palmer D.B. "Filamentous Phage Display". *Encyclopedia of life sciences*. Nature Publishing Group. http://www.els.net/ (2002).
71 - Laemmli U. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". *Nature*, 227, 680-685 (1970).
72 - Palmer D. B., Crompton T., Marandi M. B., George A. J., and Ritter M. A. "Intrathymic function of the human cortical epithelial cell surface antigen gp200-MR6: single-chain antibodies to evolutionarily conserved determinants disrupt mouse thymus development". *Immunology*, 96, 236–245 (1999).
73 - Lindenbach B.D., Evans M.J., Syder A.J., Wolk B., Tellinghuisen T.L., Liu C.C., Maruyama T. et al. "Complete replication of hepatitis C virus in cell culture". *Science*, 309, 623-626 (2005).
74- Tellinghuisen T.L., Foss K.L., Treadaway J. C. "Regulation of hepatitis C virion production via phosphorylation of the NS5A protein". *PLOS Pathogens* 4 (3), (2008).
75 - Bartholeyns J. et al. "Explantation of the observation of pancreatic ribonuclease activity at pH 4.5". *Int. J. Pept. Protein Res*., 10, 172-175 (1977).
76 - Richele L. Abel, Marcia C. Ronald T. Raines. "Fluorescence Assay for the Binding of Ribonuclease A to the Ribonuclease Inhibitor Protein". *Analytical Biochemistry*, 306, 100–107 (2002).

77 - Johnson J. et al. “Inhibition of human pancreatic ribonuclease by the human ribonuclease inhibitor protein”. J. Mol. Biol. 368, 434-449 (2007).
78 - Alter M.J., Kruszon-Moran D., Margolis H.S. “The prevalence of hepatitis C virus infection in the United States, 1988 through 1994”. *N. Engl. J. Med*., 341, 556–562 (1999).
79 - Montalto M., Cervello L., Giannitrapani F., Dantona A., Terranova L.A., Castagnetta. “Epidemiology, risk factors, and natural history of hepatocellular carcinoma”. *Ann. N. Y. Acad. Sci*., 963, 13–20 (2002).
80 - Brekke O.H. and Sandlie I. “Therapeutic antibodies for human diseases at the dawn of the twenty-first century”. *Nat. Rev. Drug. Discov*., 2 (1), 52-62 (2003).
81 - Adams G.P., and Weiner L.M. “Monoclonal antibody therapy of cancer”. *Nat. Biotechnol*., 23 (9), 1147-57 (2005).
82 - Imai K., and Takaoka A. “Comparing antibody and small-molecule therapies for cancer”. *Nat. Rev. Cancer*, 6 (9), 714-27 (2006).
83 - Fuh G. “Synthetic antibodies as therapeutics”. *Expert Opin. Biol*. Ther., 7(1), 73-87 (2007).
84 - Joly V. et al. “Enfuvirtide: from basic investigations to current clinical use”. *Expert Opin. Pharmacother*, 11 (16), 2701-13 (2010).
85 - Ghosh R.K., Ghosh S.M., and Chawla S. “Recent advances in antiretroviral drugs”. *Expert Opin Pharmacother*, 12 (1), 31-46 (2011).
86 - Shadman K.A. and Wald E.R. “A review of palivizumab and emerging therapies for respiratory syncytial virus”. *Expert Opin. Biol. Ther*., 11 (11), 1455-67 (2011).
87 - Catanese M.T., Ansuini H., Graziani R., Huby T., Moreau M., Ball J.K., Paonessa G., Rice C.M., Cortese R., Vitelli A. et al. “Role of scavenger receptor class B type I in hepatitis C virus entry: kinetics and molecular determinants”. *J. Virol*., 84, 34-43 (2010).
88 - Meuleman P., Catanese M.T., Verhoye L., Desombere I., Farhoudi A., Jones C.T., Sheahan T., Grzyb K., Cortese R., Rice C.M., Leroux-Roels G., Nicosia A. “A human monoclonal antibody targeting scavenger receptor class B type I precludes hepatitis C virus infection and viral spread in vitro and in vivo”. *Hepatology*, 55 (2), 364-72 (2012).
89 - Samuel D., Bismuth A., Mathieu D., et al. “Passive immunoprophylaxis after liver transplantation in HBsAg-positive patients”. *Lancet*, 337, 813–815 (1991).
90 - Boeckh M., Berrey M.M., Bowden R.A., et al. “Phase 1 evaluation of the respiratory syncytial virus-specific monoclonal antibody palivizumab in recipients of hematopoietic stem cell transplants*”. J. Infect. Dis*., 184, 350–354 (2001).
91 - Leyland-Jones B., Gelmon K., Ayoub J.P., et al. “Pharmacokinetics, safety, and efficacy of trastuzumab administered every three weeks in combination with paclitaxel”. *J. Clin. Oncol*., 21, 3965–3971 (2003).
92 - Troise F., Cafaro V., Giancola C., D’Alessio G., De Lorenzo C. “Differential binding of human immunoagents and herceptin to the ErbB2 receptor”. *FEBS J*., 275 (20), 4967–79 (2008).
93 - De Lorenzo C., Cozzolino R., Carpentieri A., Pucci P., Laccetti P., D’Alessio G. “Biological properties of a human compact anti-ErbB2 antibody”. *Carcinogenesis*, 26, 1890–5 (2005).