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Monoclonal Antibodies for the CIGB-552 Antitumor Synthetic Peptide Quantification

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Abstract

The CIGB-552 peptide is a synthetic peptide that exerts cytotoxic effect on tumor cells. Thus, CIGB-552 peptide quantification in patient samples is crucial for assessing the treatment efficacy. Therefore, this study describes the generation and characterization of monoclonal antibodies (mAb) directed against the CIGB-552 peptide to be used in a quantification assay in further pharmacokinetic studies in humans. In this sense, the CIGB.552-H294 mAb specificity was evidenced only against the CIGB-552 peptide and a metabolite of 17 amino acids resulting from the CIGB-552 peptide degradation detected in mouse sera. The CIGB-552 peptide quantification, challenging human plasma and mouse serum with a CIGB-552 peptide-Biotin, evidenced a decrease from 25% to 32% in the CIGB-552 peptide amount in just one min of incubation in both biological samples; and up to 8.0% in the human plasma and 46.4% in the mouse serum at 90 min, respectively. In conclusion, an immuno-quantification system based on the CIGB.552-H294 mAb is specific and sensitive and thus could be useful to quantify the CIGB-552 peptide in human plasma of patients with cancer treated with the CIGB-552 peptide.

Keywords: Cancer; Monoclonal Antibody; Synthetic Synthetic Peptide

Introduction

Cancer is a group of diseases involving abnormal cell growth with the potential to invade other parts of the body. Over 100 types of cancers affect humans. Regarding cancer causes, tobacco use and obesity are the cause of about 22% and 10% of cancer deaths, respectively. Poor diet, lack of physical activity or excessive drinking of alcohol have been also linked to cancer development. Other factors also include exposure to ionizing radiation and environmental pollutants. In the developing world, about 15% of cancers are due to infections such as Helicobacter pylori, Hepatitis B, Hepatitis C, Human papillomavirus, Epstein–Barr virus and human immunodeficiency virus. These factors act, at least partly, by changing the genes of a cell [1-4]. The World Health Organization estimates that in 2018, cancer new cases will exceed 18 million and about 9.6 million will die from this disease worldwide [5]. Currently cancer treatments involve surgery, chemotherapy, radiation; and biological and hormonal therapy. However, the high deleterious and life-threatening side effects of these interventions (chemotherapy, radiation), along with the development of resistance in patients are considered serious limitations of this therapeutic scheme [6-8]. In this sense, therapeutic peptides are a promising and a novel approach to treat many diseases including cancer. As rule, therapeutic peptides have several advantages over proteins or antibodies: as they are easy to synthesize, have a high target specificity and selectivity, high biological activity, low immunogenicity, good biocompatibility, low toxicity, great sequence diversity and a cost-effective synthesis [9-12].

Among these alternative therapeutic drugs; the CIGB-552 peptide is a hopeful approach for the development of anti-cancer treatments. The CIGB-552 peptide is an anti-tumor synthetic peptide with a high cell penetrating capacity developed through an alanine scanning from the region 32-51 aminoacids of the LALF protein of *Limulus* sp [13,14]. It exerts an anti-tumor effect through the stabilization and accumulation of the copper metabolism MURR1 domain-containing protein 1 (COMMD1 protein), which induces the ubiquitination of RelA and inhibits the anti-apoptotic activity regulated by NF-κB. It also induces peroxidation of proteins and lipids specifically in tumor cells. In addition, the subcutaneous administration of the CIGB-552 peptide promotes a significant delay on tumor growth in both syngeneic mouse tumors and patient-derived xenograft models; and it has also proved to be effective in dogs with naturally occurring cancer [14-16].

Recently, the CIGB-552 peptide was approved for performing clinical trials by the National Medicine Control Authority, nevertheless the isolation and quantification of the CIGB-552 peptide in patient samples is still an unsolved issue, which prevents the assessment of the efficacy of the treatment with the CIGB-552 peptide.

In general, peptide quantification studies have been done by combining the high performance liquid chromatography with mass spectrometry, but CIGB-552 peptide quantification previous studies performed applying these techniques failed. Perhaps, because the CIGB-552 peptide precipitation together with plasma proteins, most likely due to the peptide enrichment chemical procedures applied. In addition, the relative low short half-life of peptides and the complexity of blood samples constitute remarkable constrains to achieve a high selectivity and sensitivity in the peptide quantification assays [17]. To overcome these constrains, the combination of immunological quantification and mass spectrometry has shown a higher potential for peptide quantification [18], nevertheless, against this approach, there is no monoclonal antibodies (mAb) specific for the CIGB-552 peptide available so far.

Therefore, the subject of this paper was describing the generation and characterization of mouse mAb specific for the CIGB-552 peptide to be applied in an immuno-quantification assay of the CIGB-552 peptide in further pharmacokinetic studies in humans.

Materials and Methods

CIGB-552 Peptide Synthesis

Peptides and active peptide metabolites described in Table 1 were synthesized manually on Fmoc-AM-MBHA resin by a stepwise solid-phase procedure using the Fmoc/tBu strategy [14,19]. For biotinylated peptides, Biotin was added at the N-terminal of peptides during the solid phase synthesis. The CIGB-552 peptide was conjugated to both BSA and KLH carrier proteins using the succinic anhydridecarbodiimide method as it was previously described [20]. The carrier proteins (5 mg) were dissolved in 1 mL of 150 mM phosphate buffered saline solution (PBS), pH 8.5 and 0.5 mg of succinic anhydride. The solution was stirred during 30 min at room temperature and the free succinic anhydride was removed by gel filtration chromatography on a PD10 column loaded with Sephadex G-25 (General Electric Healthcare, USA) and equilibrated with PBS, pH 5.0. Then, 10 mg of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) were added as conjugation reagent dissolved in 500 µL of PBS, pH 5.0 [20]. The solution was stirred again for 10 min at 4 °C and the free EDC was also separated by gel filtration chromatography on a PD10 column loaded with Sephadex G-25 (General Electric Healthcare, USA) equilibrated with PBS, pH 7.2. Subsequently, 5 mg of the CIGB-552 peptide dissolved in PBS, pH 7.2 were added to the activated carrier protein to be stirred during 3 h at room temperature. Finally, the free peptides were separated from the peptide-protein conjugates by dialysis against PBS, pH 7.2 at 4 °C. The sequence and molecular weight of the CIGB-552 peptide and active CIGB-552 peptide metabolites resulting from the peptide degradation in mouse serum are described in Table 1.

Mouse Immunization Protocol

Briefly, six, randomly numbered, female Balb/c mice (22 ± 2 g) were subcutaneously immunized with 100 µg of the KLH-CIGB-552 peptide conjugated (200 µL per animal) emulsified in Freund´s adjuvant once a week during four weeks. The first administration was done in Freund's complete adjuvant, while the remaining administrations were done in Freund´s incomplete adjuvant. Partial blood extractions were done after the respective immunizations for measuring the antibody titer against the CIGB-552 peptide.

Estimation of Antibody Titers in Mice Immunized with the CIGB-552 Peptide

Polystyrene microtiter plates (Costar, USA) were coated with 10 µg/mL of the BSA-CIGB-552 peptide conjugated in $NaCO_3$/$NaHCO_3$, pH 9.6 overnight at 4 °C. Then, plates were washed three times with PBS, pH 7.2/0.1% Tween-20 (washing solution) and blocked with 1% powder milk (w/v) in PBS, pH 7.2 for 2 h at 37 °C. Next, plates were washed again 4 times with the washing solution and samples diluted in PBS, pH 7.2/0.1% Tween-20/0.01% BSA were added and incubated 1h at 37 °C. Then, plates were washed five times with the washing solution and an anti-mouse gamma immunoglobulin-peroxidase preparation (Sigma Aldrich, USA) was used to detect antibodies specific for the CIGB-552 peptide. Plates were washed again six times with the washing solution before adding the peroxidase substrate solution (3, 3, 5, 5 tetramethylbenzidine (TMB)). The enzymatic reaction was finally stopped after 10 min with 0.5 M H_2SO_4 and the absorbance was measured at 450 nm in a microplate reader Multiskan Go (Thermo-Scientific, Finland). A preimmune mouse serum and BSA were used as negative controls.

Monoclonal Antibody Generation and Production

The generation of mAb was performed by the hybridoma technology [21]. Spleen cells from the mouse with the highest antibody titer against the CIGB-552 peptide were fused with the X63-Ag8.653 mouse myeloma cells. For such purpose, spleen cells were washed twice with RPMI 1640 medium, and then polyethylene glycol (PEG) 1500 (Sigma-Aldrich, USA) was added slowly to the cell pellet under continuous shaking. Fused cells were washed with RPMI medium and distributed in 96-well plates to proceed with the cell selection in HAT-RPMI medium (Sigma- Aldrich, USA). Next, hybridoma supernatants were screened by ELISA against the CIGB-552 peptide and positive clones were cloned up to four times by limiting dilution method [22]. After respective screenings, one hybridoma was selected to produce enough amount of mAb specific for the CIGB-552 peptide in mice previously irritated intraperitoneally with liquid petrolatum. Ten days after the peritoneal cavity irritation, one million of cells per animal

were inoculated intraperitoneally to produce ascites reach in specific antibodies. Finally, animals were euthanized following recommendations for the care and use of laboratory animals [23].

Screening of Hybridoma Clones Specific for the CIGB-552 Peptide by ELISA

Polystyrene microtiter plates (Costar, USA) were coated with 10 µg/mL of the BSA-CIGB-552 peptide conjugated in $NaCO_3$/ $NaHCO_3$, pH 9.6 for 45 min at 37 °C. Plates were washed with PBS, pH 7.2/0.05% Tween-20 (washing solution) and blocked for 1 h at 37 °C with 1% powder milk (w/v) dissolved in the washing solution. Plates were washed four times and 100 µL of hybridoma culture supernatants were added to the correspondent wells to be incubated for 1h at 37 °C and washed five times with the washing solution. The antigen-antibody interaction was detected using an anti-mouse gamma immunoglobulin horseradish peroxidase preparation diluted in PBS, pH 7.2 for 1h at 37 °C. Immediately after, plates were washed again six times. Subsequently, the enzymatic reaction was revealed with O-phenylenediamine (OPD) (Sigma Aldrich, USA) substrate and stopped with 2.5 M H_2SO_4. The absorbance was measured at 492 nm using a microplate reader Multiskan Go (Thermo-Scientific, Finland).

Monoclonal Antibody (CIGB.552-H294 mAb) Purification

The mAb specific for the CIGB-552 peptide was purified from ascites by affinity chromatography using Protein A-Sepharose Fast Flow (General Electric Healthcare, USA) according to the manufacturer's instructions. Briefly, Protein A-Sepharose FF column was equilibrated with 1.5 M Glycine/3M NaCl, pH 8.9. Then, the filtered ascites fluid was mixed 1: 2 with the equilibrium solution and applied to the column at 3.3 mL/min. The elution was performed using 100 mM citric acid, pH 6.0. Finally, another column loaded with Sephadex G-25 (General Electric Healthcare, USA) was used to exchange the buffer of antibody elution fraction to 20 mM Tris/150 mM NaCl, pH 7.2.

Protein Quantitation

Protein quantification was done by Bradford method using Quick Start™ Bradford Protein Assay kit (Bio-Rad, USA) according to the manufacturer's instructions [24].

Monoclonal Antibody (CIGB.552-H294 mAb) Purity Estimation

To estimate the purity and visualize light and heavy chains of the purified mAb, a 12.5% SDS-PAGE was performed, where 20 µg of the purified mAb were mixed in 20 µL of sample buffer [25]. Separated proteins were stained with Coomassie blue R-250. Next, the reading and integration of protein bands were performed by densitometry analysis using a Scanner BIO-RAD GS-800 (Bio-Rad, USA) and the Software Quantity One ver. 4.6.9. Samples of the 12.5% SDS-PAGE gel but without staining were also transferred to a nitrocellulose membrane and immunoblotted using a peroxidase-goat anti-mouse immunoglobulin conjugated (Sigma Aldrich, USA) and diaminobenzidine as substrate for identification of gamma [26].

Specificity Determination of the Purified CIGB.552-H294 mAb for the CIGB-552 Peptide

An indirect ELISA was also used for determining the specificity of the purified mAb, where recognition of the mAb by the CIGB-552 peptide was also compared to the recognition of the CIGB-552 active metabolites resulting from the CIGB-552 peptide degradation in a mouse serum (Table 1). For such purpose, a microtiter plate was coated with 100 ng/mL of the purified mAb diluted in $NaCO_3$/$NaHCO_3$, pH 9.6 overnight at 4 °C. Later, the plate was washed three times with PBS, pH 7.2/0.1% Tween-20 (washing solution) and blocked with 1% powder milk (w/v) dissolved in PBS, pH 7.2 for 2 h at 37 °C. Then, plate was washed four times with the washing solution and peptide samples were incubated 90 min at 37 °C. For this, standard curves of the CIGB-552 peptide, and the CIGB-552 peptide metabolites conjugated with Biotin were prepared by making twelve serial dilutions 1:2 (starting at a concentration of 10 µg/mL) in PBS, pH 7.2/0.1% Tween-20/0.01% BSA. A Biotin standard curve was included as negative control. Then, the plate was washed five times with the washing solution and a goat polyclonal antibody specific for Biotin conjugated with horse radish peroxidase (Sigma Aldrich, USA) was used to detect antibodies specific for the CIGB-552 peptide and the CIGB-552 peptide metabolites. Finally, the plate was washed six times with the washing solution and the substrate solution consisting on TMB was added. After ten minutes, the enzymatic reaction was stopped with 2.5 M H_2SO_4 and the absorbance was measured at 450 nm in a microplate reader Multiskan Go (Thermo-Scientific, Finland).

Peptides	**CIGB-552**	**P17aa**	**P14aa**	**P10aa**
Sequence	HARIKPTRRLKWKYKGKFW	IKPTFRRLKWKYKGKFW	IKPTRRLKWKYKG	IKPTFRRLW
Molecular Weight (g/mol)	2686.42	2280.17	1820.19	1343.94
EC50 (ng/mL)	33.25	101.6	10110	13770
R^2	0.97	0.96	0.96	0.89

EC50, peptide effective concentration

Table 1: Characteristics of the CIGB-552 peptide and CIGB-552 peptide metabolites resulting from the CIGB-552 peptide degradation in a mouse serum

Estimation of the Purified CIGB.552-H294 mAb Association Constant (K_{aff})

The plate was coated with 10; 5; 2.5; 1.25 and 0.6 µg/mL BSA-CIGB-552 peptide conjugated in $NaCO_3/NaHCO_3$, pH 9.6 overnight at 4 °C. Additionally, a recovering with BSA (2.5 µg/mL) was used as negative control. Next, the plate was then washed five times with PBS, pH 7.2/0.1% Tween-20 (washing solution) and blocked for 1 h at 37 °C with powder milk 1% in PBS, pH 7.2/0.05 % Tween-20. The purified mAb curves were prepared by making ten serial dilutions 1: 2 (starting at a concentration of 2 µg/mL) in PBS, pH 7.2/0.05% Tween 20 incubated for 1h at 37 °C. Next, the plate was washed five times with the washing solution and an antibodies anti mouse immunoglobulin conjugated with horse radish peroxidase (Sigma Aldrich, USA) was added. The plate was washed again six times with the washing solution and TMB substrate solution was added. After ten minutes, the enzymatic reaction was stopped with 0.5 M H_2SO_4 and absorbance was measured at 450 nm in a microplate reader Multiskan Go (Thermo-Scientific, Finland). The K_{aff} was estimated using the formula K_{aff} = n-1/n [2 (Ab') - Ab] [27].

Protocol for measuring the Amount of the CIGB-552 Peptide Conjugated to Biotin in Human Plasma and Mouse Serum

Samples of the lyophilized CIGB-552 peptide conjugated to Biotin were firstly dissolved in water for injection and applied in 15 mL-corning tubes with 4 mL of PBS, pH 7.2, human plasma and mouse serum, respectively. The final concentration of the conjugated was 100 µg/mL to be incubated at 37 °C during 1, 15, 30, 45, 60 and 90 min, respectively. After respective incubation times, samples were frozen at -20 °C until the moment of quantification. The experiment was repeated twice.

Quantification of the CIGB-552 Peptide in Human Plasma and Mouse Serum

To quantify the stability of the CIGB-552 peptide in human plasma and mouse serum, a polystyrene microtiter plate (Costar, USA) was coated with just 100 ng/mL of the purified mAb diluted in $NaCO_3/NaHCO_3$, pH 9.6 overnight at 4 °C. Later, the plate was washed three times with PBS, pH 7.2/0.1% Tween-20 (washing solution) and blocked with powder milk 1% (w/v) dissolved in PBS, pH 7.2 for 2 h at 37 °C. Next, plates were washed four times with the washing solution and samples were incubated 90 min at 37 °C. A standard curve of the CIGB-552 peptide conjugated with Biotin was prepared by making twelve serial dilutions 1: 2 (starting at a concentration of 10 µg/mL) in PBS, pH 7.2/0.1% Tween-20/0.01% BSA. Then, plate was washed five times with the washing solution and a preparation of an anti-Biotin antibody conjugated with horse radish peroxidase (Sigma Aldrich, USA) was added. Finally, the plate was washed six times with the washing solution and the substrate solution consisting on TMB was added. After ten minutes, the enzymatic reaction was stopped with 2.5 M H_2SO_4 and the absorbance was measured at 450 nm in a microplate reader Multiskan Go (Thermo-Scientific, Finland). The negative controls used were human plasma, mouse serum and PBS, pH 7.2.

Statistical Analysis

The titration of antibodies specific for the CIGB-552 peptide in the mouse serum and ascites was expressed as mean and standard deviations. The specificity of the purified mAb for the CIGB-552 peptide and metabolites resulting from the CIGB-552 peptide degradation in mouse sera was estimated using non-linear regression analysis, where the EC50 measures the amount of peptide necessary to bind half of mAb. Data were expressed as the mean ± SD of triplicates of one representative experiment. Three independent experiments were performed in all cases. Statistic calculations were done using GraphPad Prism version 6.0 software (GraphPad Software, Inc). The confidence interval used in all cases was 0.05.

Results

Titration of Antibodies Specific for the CIGB-552 Peptide in Mouse Sera

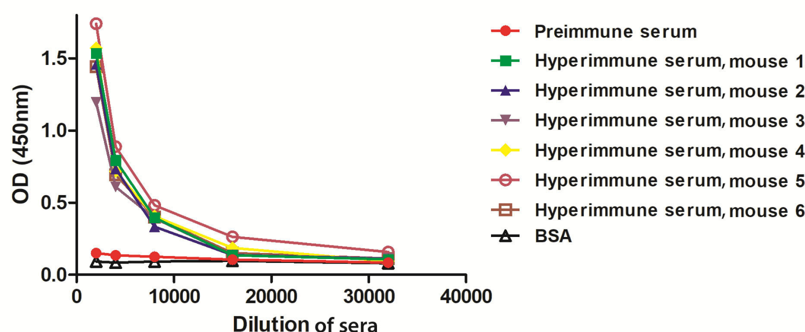

Figure 1: Results of the antibody titers in sera obtained from mice immunized with the KLH-CIGB-552 peptide conjugated measured by ELISA. Preimmune serum and BSA were used as negative controls

Regarding animal immunization and serum titration results, sera of mice subcutaneously immunized with the KLH-CIGB-552 peptide conjugated were extracted over the course of immunization for measuring the specific reactivity against the CIGB-552

peptide by ELISA. In that sense; the end-point titer was defined by the serum dilution that gave an absorbance reading higher than the pre-immune serum absorbance (≥ Average Absorbance ± 3 x Standard Deviation). This result allowed also choosing the mouse with the highest end-point titer for performing the subsequent cell fusion by hybridoma technology. As Figure 1 illustrates, the behavior of sera of all immunized mice exhibited a high end-point antibody titer against the BSA-CIGB-552 peptide conjugated used to sensibilize the ELISA plate (>1: 16000), which indicated a high immunogenicity capacity of the KLH-CIGB-552 peptide conjugated in mice. In more specific terms, the mouse with the highest end-point titer against the BSA-CIGB-552 peptide conjugated was the mouse number five showing and end-point titer equal to 1: 20000 in just 35 days after the first immunization. On the other hand, reaction of mouse preimmune sera against BSA was not observed in any case.

Generation and Purification of mAb Specific for the CIGB-552 Peptide

Spleen cells from the mouse number five were fused with the X63-Ag8.653 myeloma cells and culture supernatants from wells with growing hybridomas were screened by ELISA [28]. As it was expected from the titration analysis of the mouse sera, the screening revealed the generation of several hybridoma clones, where one stable hybridoma clone (after three cloning steps) producing of a mAb molecule specific for the CIGB-552 peptide and designated as CIGB.552-H294 showed the highest absorbance values in the ELISA (Absorbance ≥ 1.5). After this selection step, several Balb/c mice were inoculated intraperitoneally with cells of the H294 hybridoma to induce ascites production reach in the CIGB.552-H294 mAb (Figure 2A). The titration of the ascites also evidenced a recognition capacity of the BSA-CIGB-552 peptide conjugated, used to cover the ELISA plate, up to 1: 256 000 dilution. Next, ascites corresponding to the mice inoculated with H294 hybridoma was purified by affinity chromatography using a Protein A-Sepharose Fast Flow column. The Protein A-Sepharose purification uses the high affinity of *Staphylococcus aureus* Protein A to the immunoglobulin Fc domain, which allows to eliminate the bulk of the serum proteins from the ascites [29]. Results of the CIGB.552-H294 mAb purification by Protein A-Sepharose Fast Flow affinity chromatography demonstrated that the purified mAb successfully recognizes the BSA-CIGB-552 peptide conjugated up to a titer of 1: 256000 and yielded 1.3 mg of mAb/mL of ascites. Additionally, the SDS-PAGE and immunoblotting analysis showed three majoritarian bands, two at 50 kDa and one at 25 kDa, indicating the heavy and light chains of the purified mAb, respectively (Figure 2B and C). The corresponding densitometry analysis confirmed a level of purity over 94%; corroborating that the CIGB.552-H294 mAb was successfully purified, which is useful for further characterization experiments.

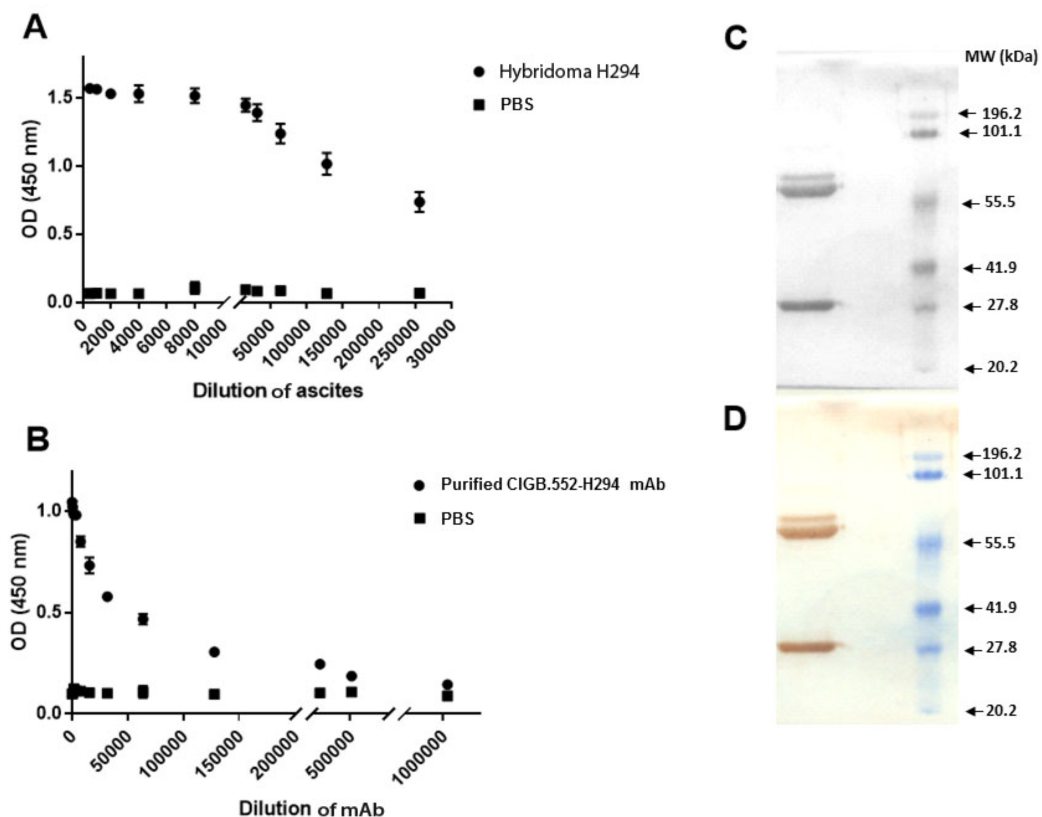

Figure 2: Illustration of the CIGB.552-H294 mAb titration. (A) Titer of the ascites produced by mice inoculated intraperitoneally with the H294 hybridoma clone. (B) Titer of the CIGB.552-H294 mAb purified by affinity chromatography using Protein A-Sepharose FF. (C) Results of the SDS- PAGE purity of the purified CIGB.552-H294 mAb. Line 1, purified CIGB.552-H294 mAb. Line 2, Molecular Weight marker (70 kDa, 66 kDa, 49 kDa, 36 kDa, 20 kDa, 14 kDa). (D) Western-blot analysis results of the purified CIGB.552-H294 mAb

Results of the Specificity Analysis of the CIGB.552-H294 mAb by the CIGB-552 Peptide

The specificity of the purified CIGB.552-H294 mAb by the CIGB-552 peptide was assessed by ELISA. With this aim, reactivity of the mAb molecule for the CIGB-552 peptide and three other peptides resulting from the CIGB-552 peptide degradation observed in mouse serum was calculated (Table 1). As shown in Figure 3, the CIGB.552-H294 mAb specifically recognizes the CIGB-552 peptide. This mAb also recognized the CIGB-552 peptide metabolite of 17 aminoacids, but in a lower extent (three folds lower EC50). The lowest signals of the CIGB.552-H294 mAb were shown for CIGB-552 peptide metabolites of 14 aminoacids and 10 aminoacids, respectively. For these two metabolites, the antibody preparation needed the highest concentrations to bind half of the concentration that gave the half-maximal response. The CIGB.552-H294 mAb recognition of Biotin and 150 mM PBS/Tween-20 0.1%/0.01% BSA used, as negative controls, was imperceptible. These results allow affirming that the CIGB.552-H294 mAb antigen recognition site is in the carboxyl terminal region of the CIGB-552 peptides (KFW).

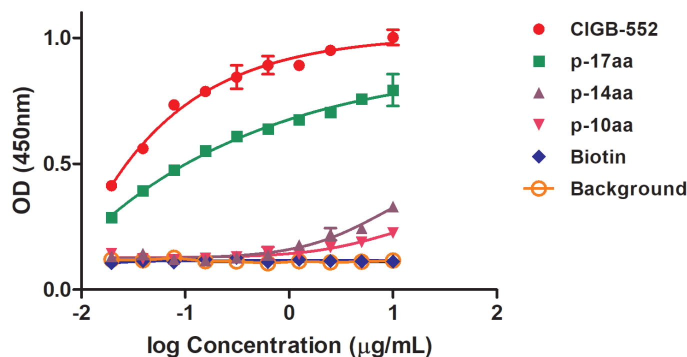

Figure 3: Illustration of the CIGB.552-H294 mAb specificity by the CIGB-552 peptide and metabolites resulting of the CIGB-552 peptide degradation in a mouse serum (Table 1). The Biotin and PBS, pH 7.2/Tween-20 0.1%/BSA 0.01% (background) were used as negative control

Results of the CIGB.552-H294 mAb Association Constant Estimation

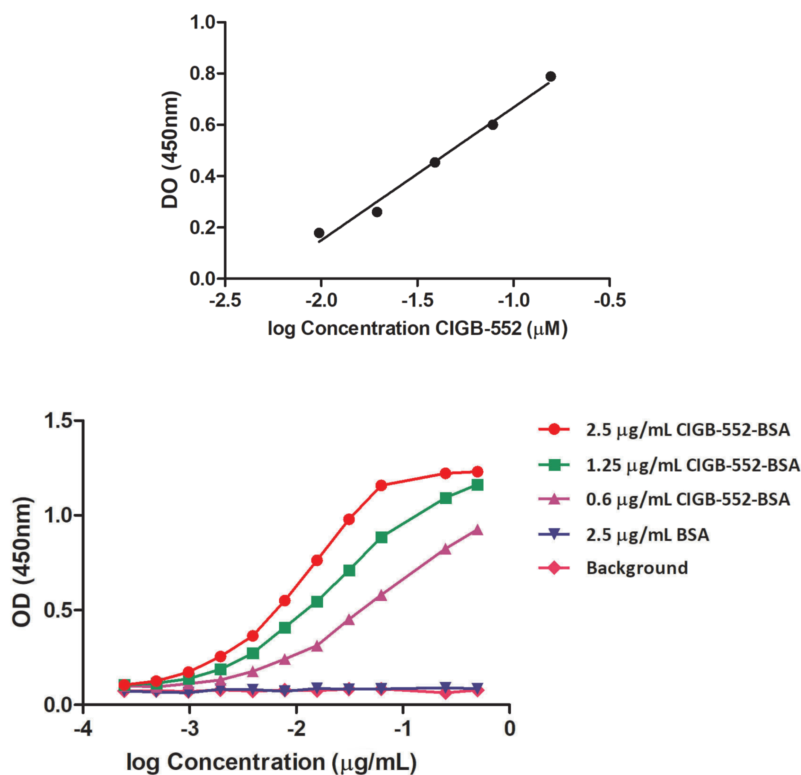

Figure 4: Results of the Kaff determination of the CIGB.552-H294 mAb by the CIGB-552 peptide. A. Lineal recognition range of the CIGB.552-H294 mAb by the CIGB-552 peptide. B. Example of the sigmoidal curves of the ELISA covering the plates at different of the BSA-CIGB-552 peptide conjugated concentration. The BSA (at 2.5 μg/mL) was used as negative control

Once the specificity of the CIGB.552-H294 mAb was clarified, authors proceeded to estimate the K_{aff} of the CIGB.552-H294 mAb by the CIGB-552 peptide using a procedure described above [23]. First, the lineal recognition range of the curve was from 0.0098

to 0.156 µg/mL with 0.99 as regression coefficient (Figure 4A). Initially, the plate coated with 2.5, 5.0 and 10 µg/mL of BSA-CIGB-552 conjugated exhibited the same behavior due to signal saturation (data not shown). Reason why, only 2.5, 1.25 and 0.6 µg/mL of BSA-CIGB-552 conjugated where chosen for coating the plates in the subsequent analysis. In details, BSA (2.5 µg/mL) was used as negative coating control and the curves corresponding to 1.25 and 0.6 µg/mL of the BSA-CIGB-552 conjugated were used to calculate K_{aff} of the CIGB.552-H294 mAb. As results, the estimated CIGB.552-H294 mAb K_{aff} was 0.9 x10^{-10} M, which indicated a relative strong K_{aff} for the CIGB-552 peptide (Figuire 4B) as a proper attribute for measuring the CIGB-552 peptide in pharmacokinetic studies. This relative high K_{aff} corresponds with an immunization schedule using several antigen doses over time.

Quantification of the CIGB-552 Peptide in Human Plasma and Mouse Serum

To examine the behavior of the CIGB-552 peptide amount in human plasma and mouse serum, 100 µg/mL of the CIGB-552 peptide conjugated to Biotin, by the amino terminal region, were incubated 1, 15, 30, 45, 60 and 90 minutes in both biological samples individually. As it can be seen in Table 2 and Figure 5, a significant decrease ($p<0.05$) in the amount of CIGB-552 peptide was observed in the initial moment of mixture [6.47% (PBS), 31.9% (human plasma), 24.9% (mouse serum)]. Then, degradation of the CIGB-552 peptide was not detected in PBS, pH 7.2 and in human plasma in 90 min of incubation (0.27% and 5.42%, $p>0.05$, respectively). On the contrary, the CIGB-552 peptide was degraded in the mouse serum [16.85% (45 min), 59.76% (90 min) $p<0.05$].

	Time (min)					
Samples	1	15	30	45	60	90
150 mM PBS, pH 7.2 + CIGB-552-Biotin (%)	93.53± 0.17	92.91±0.01	93.30±0.01	93.16±0.18	93.39±0.53	93.26±0.26
Human plasma+CIGB-552-Biotin (%)	68.10±0.85	67.71±0.80	65.70±0.60	67.96±0.29	63.82±0.55	62.68±0.20
Mouse serum +CIGB-552-Biotin (%)	75.01±0.09	71.15±0.30	70.38±1.56	58.16±1.45	49.18±0.71	40.24±1.61

Table 2: Quantification of the CIGB-552 peptide conjugated to Biotin in human plasma and mouse serum. Results are expressed as percentage of the initial amount of the CIGB-552 peptide conjugated to Biotin, *n=2*

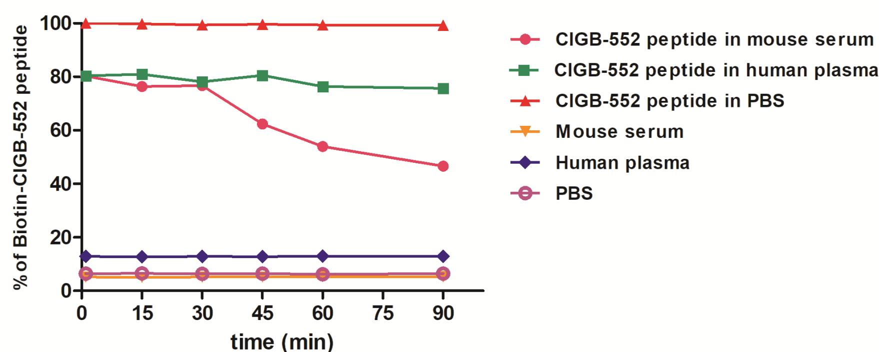

Figure 5: Results of the CIGB-552 peptide conjugated to Biotin quantification in human plasma and mouse serum from 1 to 90 min Human plasma and mouse serum were used as negative controls

Discussion

In the present study, the generation and characterization of the first mAb specific for the CIGB-552 peptide is described. The CIGB-552 peptide is a synthetic anti-tumor peptide pretended to be used as a novel therapy against cancer disease, because it exerts cytotoxic effect on tumor cells through stabilization and accumulation of the COMMD1 protein. The CIGB-552 peptide also induces the peroxidation of proteins and lipids in tumor cells, promoting a significant delay on tumor growth in syngeneic murine tumors and patient-derived xenograft models [14,15]. Therefore, the isolation and quantification of the CIGB-552 peptide in cancer patient samples during the pharmacokinetic studies performed in clinical trials is crucial for the final approval of this specific medicine.

Previous attempts done to quantify the CIGB-552 peptide by the combination of high performance liquid chromatography and mass spectrometry in human plasma and mouse serum samples failed because the precipitation of the CIGB-552 peptide together with human plasma and mouse serum proteins, most likely due to the peptide enrichment chemical procedures applied. To overcome these constrains, the use of immunological quantification shows a high potential for the CIGB-552 peptide quantification in this kind of biological samples, which impose the generation and characterization of mAb specific for the CIGB-552 peptide. To reach such goal, several hybridoma clones generated by hybridoma technology were capable of secreting antibodies directed against CIGB-552 peptide in culture supernatant. Within them, the highest secreting level was measured with the hybridoma H294.

Affinity chromatography allows the isolation of a target analyte from a complex mixture and it can be used to purify any analyte once an affinity pair is available. It involves specific interactions between a binding affinity pair, such as those between an antibody and its associated antigen. With the discovery of Staphylococcal protein A in 1970, immunoaffinity chromatography has grown in popularity and becoming now as the antibody purification gold-standard methodology [30]. Protein A is a 42 kDa surface protein present in the cell wall of the bacteria *Staphylococcus aureus.* It is composed of five homologous Ig-binding domains. Each domain is able to bind proteins from many mammalian species, mainly gamma immunoglobulins. It binds the heavy chain within the Fc region of immunoglobulins and also within the Fab region in the case of the human VH3 family. Through these interactions in serum, where IgG molecules are present, the bacterium disrupts opsonization and phagocytosis. Due to these properties, protein A has found use in biochemical research and in mAb purification processes [31].

As the application of CIGB.552-H294 mAb in the quantification of the CIGB-552 synthetic peptide in clinical trials requires antibodies with stable antigen recognition capacity and high purity, the purification based on Protein A-Sepharose Fast Flow was chose as purification method in this study. Results showed a recovery close to 80%, which is high in comparison with recoveries measured from other chromatography methods (usually below 70%) and the purity of the mAb was over 94%, which is considered lower in comparison with purity of mAb purified using for instance protein G chromatography (> 97%), but enough to the application of this mAb.

As part of the studies to characterize the purified CIGB.552-H294 mAb, a specificity assay was performed where the recognition capacity of the antibody by the CIGB-552 peptide and metabolites of the CIGB-552 peptide resulting from its degradation in a mouse serum was screened. Results revealed that the purified CIGB.552-H294 mAb molecule recognized with the highest affinity first the CIGB-552 peptide and secondly the CIGB-552 peptide metabolite of 17aa, something which is also quite convenient since these two peptides are those who only have relevant biological activity in cancer cells. Therefore, this highly specific recognition of the CIGB-552 peptide molecule allows detecting in patient samples, the amount of CIGB-552 peptide that promotes an antitumor response. This finding also indicates that the KFW amino acid triplet of the CIGC-552 peptide amino acid sequence is also necessary for the correct recognition of the CIGB-552 peptide by the CIGB.552-H294 mAb molecule. In addition, previous results have shown the importance of the KFW amino acid triplet for the interaction of the CIGB-552 peptide with its molecular target and its internalization capacity [29].

To analyze the usefulness of the CIGB.552-H294 mAb to detect the CIGB-552 peptide in complex biological samples, the CIGB-552 peptide conjugated to Biotin was incubated up to 90 minutes in human plasma and mouse serum samples. The conjugation to Biotin was done by the CIGB-552 peptide amino terminal region to quantify the CIGB-552 peptide by using antibodies specific for the Biotin in the ELISA. Results demonstrated a significant decrease of the CIGB-552 peptide amount by mixing the CIGB-552 peptide-Biotin conjugated with human plasma (25%) and mouse serum (35%) just in one minute. Authors speculate, that due to any protein precipitation procedure was not applied in the study, it could be explained by some kind of interaction of the conjugated with the human plasma and mouse serum proteins provoking interference in the quantification method or by precipitation of the conjugated after the interaction with human and serum proteins. However, after 1 min of incubation, no difference in the CIGB-552 peptide-Biotin conjugated was detected in the human plasma (<8% in 90 min), which is proper for the CIGB-552 peptide quantification in clinical trial patient samples. Inversely, a significant decrease in the amount of the CIGB-552 peptide-Biotin conjugated was detected in the mouse serum from 45 min of incubation [from 22.7% (45 min) to 46.4% (90 min)]. Therefore, further experiments have to be done to discriminate if this can be due to degradation of the CIGB-552 peptide by the CIGB.552-H294 mAb recognition site (carboxyl terminal of the CIGB-552 peptide), by an increase in the quantification interference or by precipitation of the conjugated during the incubation time. The result observed in mouse serum were coincident with those reported by Vallespí MG *et al.*, where the CIGB-552 peptide was degraded to an active metabolite of 17 aminoacids, and other two peptides of 14 amino acids and 10 amino acids, respectively in 60 min [14] (Table 1).

In regard to this, other authors have also reported a similar phenomenon. For instance, Alshammari *et al.*, obtained a low recovery of three different peptides after their incubation with sera and further quantification by mass spectrometry technique. In this work, from three tested compounds (AAHL42, AAHL18 and AAHL13), only the AALH 18 peptide could be quantified [32]. According to the authors, despite the performance of several techniques applied to eliminate the proteins from serum, the most likely cause for the low recovery of these peptides is the presence of reactive serum components that remained in the supernatant after protein purification. Though, the compound instability is a possibility that cannot be discarded. Other authors have also reported that the fast degradation of peptides before reaching the therapeutic target is a common issue considered as an important weakness of peptide-based drugs [33].

As it was mentioned before in the experiments performed with CIGB-552 peptide-Biotin conjugated, none of serum or plasma proteins were previously eliminated. Thus, the rapidly loss of the CIGB-552 peptide-Biotin conjugated within an incubation of 1 minute in both human plasma or mouse serum could be due either to binding of the CIGB-552 peptide to common plasma or serum proteins or to the interference of blood elements since the peptide could be degraded or adsorbed by them.

Regarding differences in the CIGB-552-Biotin peptide conjugated behavior in human plasma and mouse serum, Böttger *et al.*, also demonstrated that peptides are usually less stable in serum compared to plasma and fresh blood [34]. One reasonable explanation

for this phenomenon is that protease composition of serum is different to plasma since coagulation factors are active in serum but not in plasma and thus differences in their composition leads to different proteolytic profiles [35,36].

Summarizing, these results corroborate that the CIGB.552-H294 mAb is specific for the CIGB-552 peptide and its 17 amino acid metabolite and thus could be suitable to be used in an immunoassay for the CIGB-552 peptide quantification in human plasma.

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