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Binding Properties and Biological Activity of a Novel Therapeutic Monoclonal Antibody Directed toward HER2 Antigen

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ABSTRACT

Several biosimilars for Herceptin® (trastuzumab) and other innovator monoclonal antibodies (mAbs) against HER2 molecule have been developed and approved in the last years. Here, it is reported the binding properties of a novel anti-HER2 mAb (called 5G4) as well as the capacity of this mAb to inhibit cell proliferation and to induce Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC). mAb 5G4 was compared with Herceptin® in a panel of human normal tissues, breast tumors and cell lines using immunohistochemistry, immunocytochemistry, western blot, flow cytometry and colorimetric methods. mAb 5G4 showed weak to moderate staining in breast ductal cells (1/2), gastric glandular cells (1/3) and renal tubes (2/3). Additionally, an intense reactivity was evident with mAb 5G4 in HER2-positive breast adenocarcinomas. The tissue staining was slightly more intense with Herceptin® (trastuzumab) that was used as control. Flow cytometry analysis revealed that mAb 5G4 is able to react with HER2 overexpressing cells (SK-BR-3 and SKOV3) comparable to Herceptin®. The relative binding and the anti-proliferative activity ranges of mAb 5G4 using Herceptin® as reference were between 91-115% and between 91-106%, respectively, which are considered comparable. Moreover, preliminary results suggest that the capacity of mAb 5G4 to induce ADCC in SK-BR-3 cells is non-inferior to Herceptin®. Our data permit to consider that mAb 5G4 has similar binding properties to HER2 antigen and biological activity compared with Herceptin®. However, further determinations such as affinity and FcgRIIIa binding activity of mAb 5G4 could be useful to confirm these results.

Keywords: HER2 antigen; Monoclonal antibody, Binding properties, Biological activity

ABBREVIATIONS

ADCC: Antibody-Dependent Cell Cytotoxicity; AP: Alkaline Phosphatase; ATCC: American Type Culture Collection; BCIP: 5-Bromo-4-Chloro-3-Indolyl Phosphate; CD6: Cluster Of Differentiation 6; CV: Coefficient of Variation; DAB: 3,3-Diaminobenzidine; DMEM: Dulbecco's Modification Of Eagle Medium; EC50: Half-Maximal Effective Concentration; EMEM: Eagle's Minimum Essential Medium; FBS: Fetal Bovine Serum; FITC: Fluorescein Isothiocyanate; HER2: Human Epidermal Growth Factor Receptor 2; IgG: Immunoglobulin GIHC: Immunohistochemistry; kDa: KiloDalton; mAb: Monoclonal Antibody; MFI: Mean Of Fluorescence Intensity; NBT: Nitro-Blue Tetrazolium; PBS: Phosphate Buffered Saline; RPMI: Roswell Park Memorial Institute Medium; TBS: Tris-Buffered Saline

BACKGROUND

Trastuzumab (Herceptin®) is a monoclonal antibody (mAb) directed against the type 2 epidermal growth factor receptor (ERBB2, HER2 or HER-2 / neu), which is overexpressed in approximately 15-30% of invasive breast adenocarcinomas [1]. Herceptin® is used for the treatment of HER2-positive breast cancer, providing significant clinical benefits in patients with advanced or metastatic disease [2,3]. This mAb is also prescribed for the treatment of gastric adenocarcinomas with overexpression of the HER2 receptor [4].

Although effective, Herceptin®-based therapies remain expensive and inaccessible to meet the needs of many health systems. For these reasons, the development and approval of biosimilars to originator *mAb* is a necessity to reduce costs, increase health coverage and improve the quality of life of cancer patients. Given that Herceptin® patents have expired, several biosimilars for this mAb have been developed and approved in recent years (e.g. Herzuma®, Ogivri™, Ontruzant®) [5-7]. In addition, other innovative mAbs are currently in early stages of development or evaluation. In this context, a 5G4 mAb was generated at the Center of Molecular Immunology (Havana, Cuba).

The 5G4 mAb was generated by transferring a plasmid DNA encoding trastuzumab and produced by the non-secretory murine myeloma cells (NSO) expression system [8]. However, trastuzumab is produced using the Chinese Hamster Ovary (CHO) cells. Although similar conformational characteristics of 5G4 mAb and Herceptin® were previously published [8], differences in the expression system could affect both the binding properties and biological activity of mAb 5G4, due to changes in the glycosylation pattern.

In this study, cross-reactivity of mAb 5G4 in normal adult tissues is reported (this was part of the non-clinical safety assessment of 5G4). In addition, the binding properties of 5G4 mAb are presented in breast tumors, as well as in a variety of human cell lines with different levels of HER2 expression. Preliminary results are also shown regarding the ability of 5G4 to inhibit cell proliferation and induce Antibody-Dependent Cellular Cytotoxicity (ADCC) in vitro.

METHODS

Monoclonal antibodies

The anti-HER2 mAb 5G4 developed in the Center of Molecular Immunology (Havana, Cuba) was used. This humanized IgG1 mAb was produced by the non-secreting murine myeloma cells (NSO) expression system transfected by plasmid DNA encoding Herceptin®. In all assays, trastuzumab (Herceptin®, Roche) was used as reference control. Trastuzumab is produced by means of an expression system in Chinese Hamster Ovary (CHO) cells. Itolizumab, a humanized mAb specific against CD6 molecule, served as isotype-matched negative control. For immunohistochemical assays, anti-HER2 mAbs and pooled human immunoglobulins (Intacglobin®) were conjugated using the BiotinTag™ Micro Biotinylation Kit (Sigma, 1001976388 BTAG-1KT). For the evaluation of antigenic preservation of frozen tissues, a polyclonal antibody against von Willebrand factor (vWF) (Dako, A0082) was used. An anti-β-actin mAb (Sigma, A2228) was used as a loading control for western blot analysis.

Tissue samples and processing

64 samples of normal adult tissue and 5 samples of breast adenocarcinoma were obtained from the pathology department of the National Institute of Neoplastic Diseases (Lima, Peru) and the Department of Legal Medicine at the Provincial Hospital "Amalia Simoni" (Camaguey, Cuba). All samples were used after receiving the written approval of each institutional ethical committee (INEN number: 151-2017-CRP-DI-DICON/INEN; number of the Provincial Hospital "Amalia Simoni": 05-04-2000). Tissue samples were stored at -150 °C. Five micrometer sections were obtained on a cryostat and the slides were stored at -70 °C until use.

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Immunohistochemical assay

The sections were fixed in acetone for 10 minutes at 2-8 °C and rehydrated in TBS (Dako, S3001). Endogenous peroxidase activity was blocked with a commercial solution (Dako, S2003) for 10 minutes. For kidney, liver and spleen sections, endogenous biotin activity was blocked using the biotin blocking system (Dako, X0590). The tissues were then incubated for 1.5 hours with biotinylated anti-HER2 or Intacglobin® (100 µg mL-1) at room temperature. After washing with TBS, the sections were incubated with streptavidin/peroxidase (Dako, K0690) for 30 minutes. Sections of breast carcinoma positive for HER2 were used as positive controls. Enzymatic activity was visualized with a DAB solution (Dako, K3467) and the slides were counterstained with Harris hematoxylin, dehydrated and mounted.

Cell line and culture conditions

Human cell lines SK-BR-3 (HTB-30™), SKOV3 (HTB-77™), A431 (CRL-1555™), NCI-H292 (CRL-1848™), NCI-H125 (CRL-5801™), HEK-293 (CRL-1573™), HEp-2 (CCL-23™) and Ramos (CRL-1596™) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were grown according to ATCC recommendations. The DMEM (Gibco, 12800-017), RPMI-1640 (Gibco, 23400-062), EMEM (Gibco, 41500-018) and McCoy´s 5A (Sigma, M9309) media were appropriately used (Table 1). All culture media were supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Hyclone, SH30071.03). The cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2.

Western blot

The protein lysates of SK-BR-3 (cells that overexpress HER2) and Ramos (HER2 negative cells) were obtained following standard procedures. Protein samples were electrophoresed in 7.5% Bis-Tris gels and transferred by electroblotting to nitrocellulose membranes using the TE 70 Semi-Dry Transfer Unit (Amersham, United Kingdom). The transfers were blocked with 5% BSA/ 0.1% Tween-20 in TBS for 1 hour and then incubated for 1.5 hours at room temperature with respective anti-HER2 (10 µg mL-1) or anti- β-actin (1 µg mL-1) mAbs. Then, the transfers were washed and incubated with corresponding horseradish peroxidase (Sigma, A4416) or Alkaline Phosphatase (AP) (Sigma, A1543) secondary antibody bound at 1: 1000 dilutions for 1 h. Finally, the spots were washed and the enzymatic activity was visualized using DAB (Sigma, D8001) or SIGMAFASTTM BCIP®/ NBT substrate (Sigma, B5655). The molecular weight of the protein was estimated based on the values obtained for the bands in the protein standard. The images were analyzed with GeneTools ™ 4.3.8.0 software (Synoptics Ltd, United Kingdom).

Immunocytochemical assay

Cells monolayers were fixed in absolute acetone during 1.5 minutes at 2-8 ºC and rehydrated in PBS (Capricorn, PBS-1A) for 10 minutes. Samples were incubated with anti-HER2 antibodies at 10 µg mL^{-1} during 30 minutes at room temperature. After that, cells were washed with PBS for 5 minutes and incubated with FITC-conjugated rabbit anti human IgG (Sigma, F1641) 1:50 dilution for 30 minutes. Finally, cells were contrasted with propidium iodide (5 µg mL^{-1}) for 1 minute. For microscopic analysis, an Olympus inverted microscope CKX41 (Tokyo, Japan) was used for analyses.

Flow cytometric determinations

Cells (2.5×10^5) were incubated with anti-HER2 mAbs (20, 10, 5, 2.5, 1.25, 0.62, 0.31 and 0.15 µg mL^{-1}) or itolizumab (20 µg mL^{-1}) for 30 minutes at 2-8 °C. Three different batches of 5G4 mAb and two of Herceptin® were tested. Cells were washed with PBS and spun down at 268g for 10 minutes at 4 °C. Samples were incubated with FITC-conjugated rabbit anti human IgG (Dako, F0202) 1:100 dilution for 30 minutes at 2-8 °C. After that, cells were washed in PBS, resuspended and filtered using 50-µm nylon-mesh filters (Partec, NC9667434). A Beckman Coulter's Gallios cytometer containing an argon laser (488 nm) was used for binding determination.

Table 1: Cell lines and culture conditions.

Cell line	ATCC code	Origin	Culture media	Subcultivation ratio
SK-BR-3	HTB-30™	Breast adenocarcinoma	McCoy's 5A	1:02
SKOV3	HTB-77™	Ovarian adenocarcinoma	DMEM	1:2 to 1:6
A431	CRL-1555™	Skin epidermoid carcinoma	DMEM	1:3 to 1:8
NCI-H292	CRL-1848™	Mucoepidermoid lung carcinoma	RPMI-1640	1:3 to 1:8
NCI-H125	CRL-5801™	Adenosquamous lung carcinoma	RPMI-1640	1:2 to 1:6
HEK-293	CRL-1573™	Embryonic kidney	DMEM	1:6 to 1:10
HEp-2	CCL-23™	Cervical adenocarcinoma	EMEM	1:4 to 1:10
Ramos*	CRL-1596™	Burkitt lymphoma	RPMI-1640	2 x 10^5 viable cells/mL

*Suspension growth

ADCC assay

The ADCC assay was performed using the lactate dehydrogenase (LDH) detection kit (Roche, 11644793001) according to manufacturer´s instructions. Briefly, 5 000 SK-BR-3 cells (target cells, T) were incubated with 3 concentrations (1.00, 0.037 and 0.0013 µg mL^{-1}) of 5G4 mAb and Herceptin® for 30 minutes at 37 °C, 5 % CO_2 in McCoy´5A containing 1% FBS. Then, 50 µL of effector cells (peripheral mononuclear cells from a healthy volunteer donor, E) (E: T ratio 1:100) was added to each well and plates were incubated for 4 hours. Then, supernatant (100 µL) was collected and incubated with equal volume of LDH reaction mixture for 30 minutes at room temperature protected from light. Sample absorbances were measured at 490 nm, and absorbance at 620 nm was used as reference. Adequate controls for spontaneous and maximal lysis values were included to calculate the percentage of specific cytotoxicity.

Inhibition of cell proliferation

The anti-proliferative effect was determined by WST-1 (Roche, 11644807001) cell proliferation reagent. SK-BR-3 cells (10 000 cells/well) were incubated with 8 concentrations (0.001-3.0 µg mL^{-1}) of 5G4 mAb and Herceptin® for 72 hours at 37 °C, 5 % CO_2 in McCoy´5A containing 1% FBS. Then, 20 µL of WST1 reagent was added to each well and plates were incubated for 4 hours. The relative number of viable cells was quantified by measuring the absorbance of the formazan at 450 nm and 630 nm using a Spectra-Max Plus (Molecular Devices).

Statistical analysis

The Mean Of Fluorescence Intensity (MFI) for each mAb and concentration was determined using Kaluza software version 1.2 (Beckman Coulter). Curves were constructed using GraphPad Prism 4.0 (GraphPad Software Inc., USA) and PLA 2.0 (Stegmann Systems,

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Germany). The value of EC50 was calculated and the relative binding activity of 5G4 mAb using Herceptin® as standard was determined as follows: $(1/EC50_{Sample}) / (1/EC50_{Standard}) \times 100$. For anti-proliferative assays, EC50 values and relative potencies were calculated using SoftMax Pro 5.4.1 program (Molecular Devices), and for ADCC assays data were analyzed using GraphPad Prism 4.0 software. Mann Whitney U Test was used to compare the reactivity of mAbs in different cell lines. Kruskal-Wallis followed by Dunn's multiple comparison tests were used to compare the ADCC activity of mAbs at different concentrations. The criterion for statistical significance was $p < 0.05$.

RESULTS

Validation of tissue samples preservation

No significant alterations in histoarchitecture, based on hematoxylin and eosin staining, was observed in the frozen tissues panel. Moreover, an intense reactivity of the anti-vWF polyclonal antibody in the cytoplasm of vascular endothelial cells was evidenced, verifying the antigenic preservation of tissues (Figure 1).

Immunohistochemistry-based tissue cross-reactivity study of 5G4 mAb

Table 2 shows the cross-reactivity study for 5G4 mAb in a panel of normal human tissues. 5G4 mAb reacted with breast ductal cells (1/2) (Figure 2A,2B), glandular cells from stomach (1/3) (Figure 2C,2D) and renal tubes (2/3) showing a weak to moderate cytoplasmic staining. In general, the reaction pattern of 5G4 was similar, but slightly inferior in intensity compared to Herceptin®. No immunostaining with both anti-HER2 mAbs was detected in the rest of evaluated tissue samples. No reactivity was also observed in tissues samples incubated with Intacglobin®, used as negative control.

A specific, intense and linear staining of cell membrane was evidenced with 5G4 mAb in breast adenocarcinomas (Figure 2E,2F) and SK-BR-3 cells previously classified as HER2-positive (3+) (Figure 3A,3C). This pattern of staining was comparable to that obtained with Herceptin®. No reaction was detected in HER2-negative breast adenocarcinoma sections neither in Ramos cell monolayers (Figure 3B,3D). Moreover, the capacity of 5G4 mAb to specifically bind to HER2 molecule (~ 180 kDa for both) was also confirmed by western blots using cell lysates obtaining from SK-BR-3 breast cancer cell line (Figure 2E).

Reactivity of 5G4 mAb in human cell lines by flow cytometry

The binding of HER2 molecule by 5G4 mAb was also evaluated by flow cytometry in a series of tumor cell lines (Table 3). This mAb displayed similar recognition profile in HER2-overexpressing cells (SK-BR-3 and SKOV3) to Herceptin® as well as in some cells with low expression of this molecule (H292, A431 and HEK-293) (Figure 3F,3K). But, statistically significant differences in NCI-H125 ($p = 0.0022$) and HEp-2 ($p = 0.0152$) cells (that express HER2 at low levels) were observed when compared with Herceptin®. No recognition with 5G4 mAb or Herceptin® was achieved in the HER2-negative cells (Ramos).

Relative binding activity of 5G4 mAb in SK-BR-3 cells

5G4 mAb was able to binds HER2 molecule in a dose-dependence manner using SK-BR-3 breast cancer cells. The binding saturation was achieved at 10 µg mL-1 for both 5G4 mAb and Herceptin® (Figure

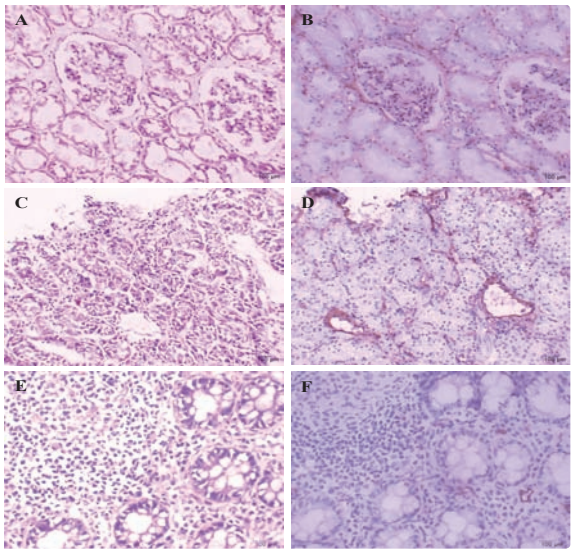

Figure 1: Representative microphotografs of normal adult tissues. A, C and E: hematoxylin and eosin staining of kidney, stomach and large intestine, respectively. B, D and F: immunohistochemical detection of blood vessels using an anti-von Willebrand factor (vWF) polyclonal antibody (brown color). Counterstaining with Harris's Hematoxylin (blue color). Black bars = 100 µm.

Table 2: Tissue cross-reactivity of mAb 5G4 compared to Herceptin®.

Tissue samples	HER2 detection	
	mAb 5G4	Herceptin®
Skin	0/3	0/3
Esophagus	0/2	0/2
Stomach	1/3+	1/3++
Epithelium	0/3	0/3
Glandular cells	1/3+	1/3++
Small intestine	0/2	0/2
Large intestine	0/3	0/3
Pancreas	0/2	0/2
Liver	0/3	0/3
Lung	0/2	0/2
Bronchi	0/1	0/1
Kidney	2/3+	2/3++
Corpuscle	0/3	0/3
Tubules	2/3+	2/3++
Urinary bladder	0/2	0/2
Ureter	0/2	0/2
Prostate	0/2	0/2
Ovarium	0/3	0/3
Breast	0/2	0/2
Testis	0/3	0/3
Uterus	0/3	0/3
Fallopian tubes	0/2	0/2
Brain	0/2	0/2
Cerebellum	0/1	0/1
Encephalic trunk	0/1	0/1
Tonsil	0/1	0/1
Spleen	0/2	0/2
Peripheral blood (white cells)	0/2	0/2
Veins, Arteries	0/3	0/3
Striated muscle	0/3	0/3
Thyroid	0/2	0/2

+weak staining; ++moderate staining

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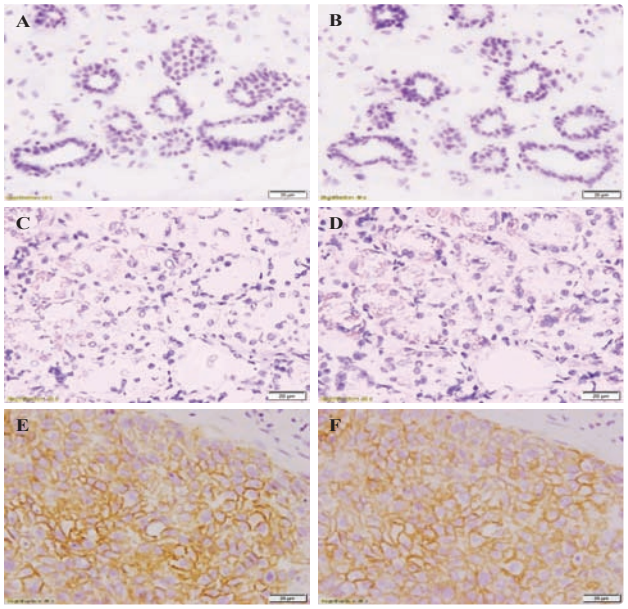

Figure 2: Tissue cross-reactivity of 5G4 mAb in comparison with Herceptin®. A and B: Representative photomicrographs of normal breast sections. Observe the lack of reactivity of 5G4 mAb and Herceptin® in ductal cells. In normal stomach sections, a very low (C) and a low-to-moderate (D) staining (brown color), mainly located in the cytoplasm of normal glandular cells from mucosal epithelium, was evidenced with 5G4 mAb and Herceptin®, respectively. HER2-positive (3+) breast adenocarcinoma sections are shown in E and F. Note the intense reaction with both anti-HER2 mAbs located in the plasma membrane of malignant epithelial cells (brown color). In all samples, nuclei were counterstained with Harris hematoxylin (blue color). Scale bar = 20 µm. 400x magnification.

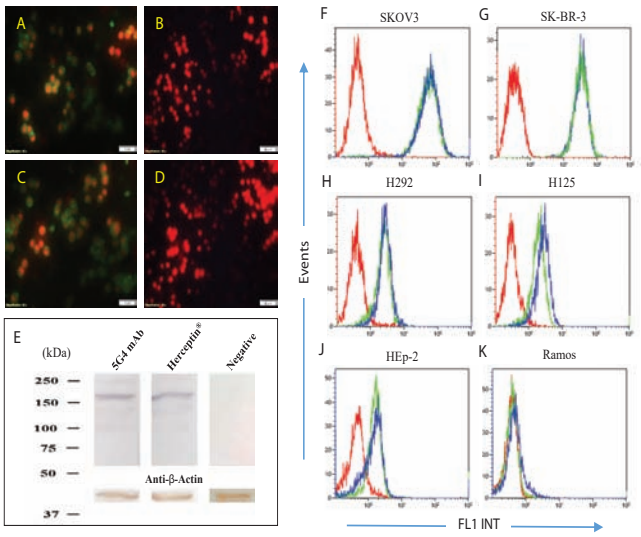

Figure 3: Binding characteristics of 5G4 mAb in comparison with Herceptin®. A-D: Reactivity of anti-HER2 mAbs in SK-BR-3 and Ramos cell monolayers by immunofluorescence. Note the intense staining of 5G4 mAb (A) and Herceptin® (C) in the plasma membrane of SK-BR-3 malignant epithelial cells (green color). In contrast, no staining with these mAbs was detected in the HER2-negative Ramos cells (B and D, respectively). Nuclei were counterstained with propidium iodide (red color). Scale bar = 20 µm. 400x magnification. E: Western blot using SK-BR-3 and Ramos cells lysates. 5G4 mAb specifically bind to HER2 molecule (~ 180 kDa) expressed in SK-BR-3 cells, similar to Herceptin®. F-K: Flow cytometry assays in different human cell lines. Red, blue and green lines represent isotype-matched negative control, 5G4 mAb and Herceptin®, respectively.

Table 3: Reactivity of mAb 5G4 and Herceptin® in human cell lines by flow cytometry.

	HER2 detection		
Cell line	**mAb 5G4**	**Herceptin®**	**P value[a]**
	MFI ± SD	MFI ± SD	
SKBR3	30.79 ± 1.28	32.52 ± 0.67	ns
SKOV3	33.51 ± 1.24	34.31 ± 0.43	ns
H292	2.49 ± 0.07	2.72 ± 0.04	ns
A431	2.48 ± 0.04	2.63 ± 0.02	ns
HEK-293	2.16 ± 0.06	2.55 ± 0.08	ns
NCI-H125	2.33 ± 0.14	2.54 ± 0.11	0.0022
HEp-2	1.35 ± 0.03	1.79 ± 0.09	0.0152
Ramos	1.46 ± 0.08	1.46 ± 0.09	ns

MFI: Mean Fluorescence Intensity; SD: Standard Deviation; ns: not significant; [a]p values were determined using Mann Whitney U Test.

4A). In all flow cytometric assays, the coefficients of variation were ≤ 16% for each tested concentration and mAb. The EC50 range of MFI was 1.765-1.821 and 1.649-2.100 µg mL-1 for 5G4 mAb and Herceptin® batches, respectively. In consequence, the relative binding activity range of 5G4 mAb was 91-115% using Herceptin® as standard (Table 4). No reaction was obtained with the isotype negative control.

Capacity of 5G4 to inhibit cell proliferation

The anti-proliferative effect of 5G4 mAb and Herceptin® on SK-BR-3 cells is shown in figure 4B. In all experiments, the coefficients of variation were ≤ 13% for each tested concentration and mAb. The maximum inhibition of cell growth of 74% and 77% was obtained with 3 µg mL^{-1} of 5G4 mAb and Herceptin®, respectively. The EC50 values for 5G4 mAb ranged from 0.078-0.295 µg mL^{-1}, similar to those obtained with Herceptin® used as control (0.077-0.316 µg mL^{-1}). The relative potency range of 5G4 mAb was 91-106% using Herceptin® as reference.

ADCC activity of 5G4 mAb

The capacity of 5G4 mAb to induce ADCC activity was evidenced in SK-BR-3 cells using peripheral mononuclear cells from healthy volunteer donors. Two batches of this mAb (2 and 3) showed non-inferior activity when compared with Herceptin® (Figure 4C). The batch 1 of 5G4 mAb showed decreased ADCC activity when compared with batch 2 of Herceptin® in all evaluated concentrations ($p = 0.0091$, $p = 0.0192$ and $p = 0.0144$, respectively). Coefficients of variation were ≤ 15% for each tested concentration and mAb. No ADCC activity was detected with negative controls.

DISCUSSION

The introduction of novel therapeutic mAbs into clinical practice requires careful characterization, including binding properties to both normal and malignant tissues during preclinical studies. Some techniques are commonly used to evaluate the antigen binding attributes of mAbs such as, Immunohistochemistry (IHC), western blot with tissue lysates and flow cytometry using cell lines that expresses the target molecule.

Tissue cross-reactivity permits the identification of both, previously unknown sites of the antigen expression and binding of mAbs to unexpected targets, such as cross-reactive epitopes, alerting oncologists to potential toxicity to certain organs that could be observed clinical trials [9]. These studies are an essential part of the

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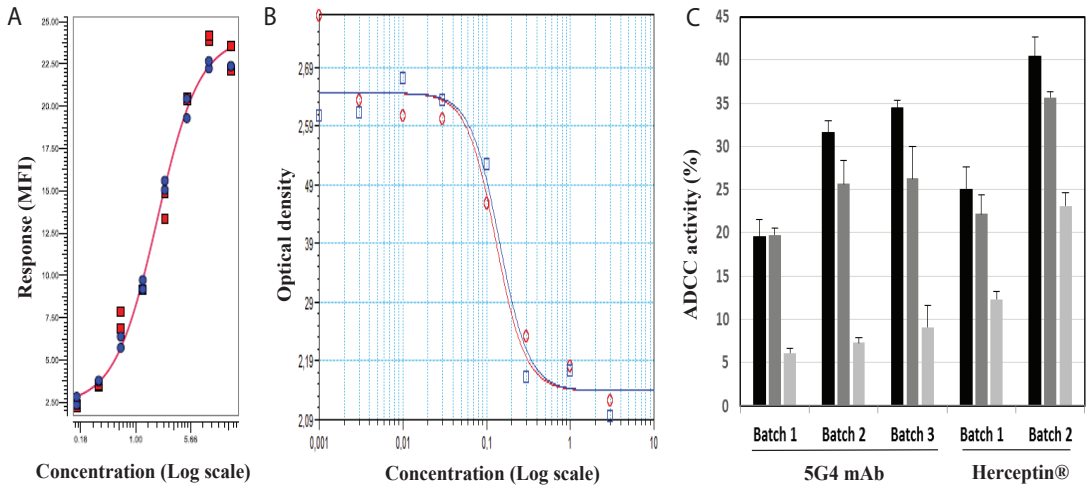

Figure 4: Biological activity of 5G4 mAb in comparison with Herceptin® in SK-BR-3 cells. A: Relative binding activity of 5G4 mAb. Observe the similar doses-response curves for this mAb (blue color) and Herceptin® (red color). B: Potency curves of 5G4 mAb obtained from the anti-proliferative assay. The capacity of 5G4 mAb (blue color) was similar to that of Herceptin® (red color). C: ADCC activity of 5G4 mAb. In general, the cytotoxicity of this mAb was non-inferior when compared with Herceptin®, except for batch 1 of 5G4 mAb that exhibited a decreased ADCC activity when compared with batch 2 of Herceptin®.

Table 4: Relative binding activity of mAb 5G4 using Herceptin® as standard.

Assay number	Experimental design	EC50 value	Relative binding activity (%)
1	Standard: Herceptin® batch 1	1.649	-
	Samples		
	Herceptin® batch 2	1.732	95
	mAb 5G4 batch 1	1.808	91
	mAb 5G4 batch 3	1.765	93
2	Standard: Herceptin® batch 1	2.059	-
	Samples		
	Herceptin® batch 2	2.1	98
	mAb 5G4 batch 2	1.821	113
1	Standard: Herceptin® batch 2	1.732	-
	Samples		
	Herceptin® batch 1	1.649	105
	mAb 5G4 batch 1	1.808	96
	mAb 5G4 batch 3	1.765	98
2	Standard: Herceptin® batch 2	2.1	-
	Samples		
	Herceptin® batch 1	2.059	102
	mAb 5G4 batch 2	1.821	115

% - Percentage.

preclinical safety assessment package of therapeutic mAbs [10,11]. The preservation of both tissue morphology and molecular antigenic determinants has to be firstly demonstrate in IHC studies [9]. Here, both morphological and antigenic tissue conservation was evidenced in all tissue samples, permitting to consider these specimens adequate for the cross-reactivity study.

In general, the expression of HER2 molecule was limited in normal adult tissues. These results are in line with previous reports concerning the low expression of HER2 molecule in normal tissues when compared with malignant tumors [1,12,13]. The pattern of recognition of 5G4 mAb was similar to those obtained with Herceptin®, although a slightly diminished in intensity, suggesting

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no dramatic differences in the affinity and/or in the specificity of this new anti-HER2 mAb. However, in this panel of frozen tissues no immunostaining with 5G4 mAb was observed in other normal epithelial cells previously reported positive to HER2 molecule (e.g. small intestine, tonsil, uterus) [12,13]. Therefore, experiments with a larger number of these tissue samples should be performed to confirm these results.

Similar to Herceptin®, an intense membrane staining with 5G4 mAb was evident in breast carcinomas that overexpress HER2 but not in HER2-negative breast tumors, which support the specificity of this mAb. This fact was also confirmed by Western blots using lysates of SK-BR-3 cells (cells that overexpress HER2). In addition, mAb 5G4 was able to react with a variety of epithelial cells positive for HER2. However, in two low-expression cell lines, the reactivity of 5G4 mAb decreased compared to Herceptin®, in concordance with the previous results obtained by IHC. More studies are needed, such as the affinity of mAb by surface plasmon resonance and/or homologous displacement curves to confirm this result.

Bioassays are key tools for product characterization and depend on the use of reference standard. The innovator (reference product) is necessary to demonstrate biosimilarity [14] and here was used as standard. The biological activity related to Fab fragments was determined both by *in-vitro* binding and by antiproliferative activity of candidate mAb and the potency estimate for each bioactivity was calculated. In the present study, the relative binding activity of mAb 5G4 using EC50 calculation ranged between 91-115% with respect to Herceptin®. These values met the acceptance criteria (80-120%) commonly used for this type of assays [15] and support the ability of 5G4 mAb to bind to HER2 antigen as well as the reference product. Similarly, the antiproliferative activity of mAb 5G4 ranged between 91 and 106 % compared to Herceptin®, values that are also considered comparable [15].

It is known, ADCC is a primary mechanism of action for Herceptin® [16]. Here, preliminary data on the ability of 5G4 mAb to also induce this mechanism of action is shown. However, a decrease in the capacity of this mAb when compared with Herceptin® was observed, although only statistically significant differences were detected for a batch of 5G4 mAb. This result could be related to differences in the glycosylation pattern of 5G4 mAb due to differences in expression systems [8,17], which affect this Fc-dependent mechanism. In fact, a decrease in both % of fucosylation and galactose-terminal was found in 5G4 mAb (unpublished data). These changes were previously related with a decrease in Herceptin® ADCC activity [16].

However, a reduction in the % of fucosylation and galactosylation was unable to affect the binding and inhibition of cell proliferation activity of Herceptin® [16], similar to the present study. In addition, differences in ADCC activities could not affect the effectiveness of Herceptin® [16]. Interestingly, the anti-tumor effects of mAb 5G4 on athymic nude mice with SKOV3 ovarian cancer xenografts were not inferior to Herceptin® (unpublished data). In general, these results suggest the potential use of 5G4 mAb for the treatment of tumors that overexpress HER2. However, the development of a new anti-HER2 mAb using CHO cells as expression system is recommended.

CONCLUSION

In summary, this study reported the binding properties and biological activity of 5G4, a novel anti-HER2 mAb. The limited reactivity of mAb 5G4 against normal adult tissues provided information to clinical oncologists about the possible low toxicity to normal organs as observed in the first clinical trials in humans. Our data allow us to consider that mAb 5G4 has binding properties similar to the HER2 antigen and biological activity compared to Herceptin®. However, other determinations such as affinity and FcgRIIIa binding activity of mAb 5G4 could be useful for confirming these results.

DECLARATIONS

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files. The histological slides are archived in the Laboratory of Recognition and Biological Activity Assays (Center of Molecular Immunology, Havana, Cuba). Archived stained slides are available from the corresponding author on reasonable request.

Authors' contributions

RB performed and analyzed the data from almost all experiments included in the study, discussed the results and was the major contributor in writing the manuscript. LAA and MR performed the tissue processing for histology and the bio repository management. EL, LC, DY and MC performed additional experiments. SC and MC performed the histological examination of normal and malignant tissues. BS designed and supervised ADCC assays. NL and MC revised and corrected the manuscript. All authors read and approved the final manuscript.

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