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Estudio de los efectos de fármacos antineoplásicos sobre células gástricas neoplásicas humanas.

Study on the effects of anticancer drugs on human gastric cancer cells.

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RESUMEN

El objetivo de este experimento es realizar un estudio comparativo sobre los efectos de la herceptina en la línea celular de cáncer gástrico HCG27. Primero, se realizaron curvas dosis-respuesta para herceptina, irinotecan y 5-FU como referencia para la efectividad de la herceptina. A continuación, se realizaron Western blots sobre diversos marcadores tumorales para ver los efectos de otros fármacos sobre HCG27. Los resultados mostraron que en una línea celular de cáncer gástrico donde HER2+ esta sobrexpresado, como es el caso de HCG27, la herceptina muestra una clara actividad anticancerígena.

ABSTRACT

The main objective of this experiment was to compare and study the effects of herceptin, an anticancer drug, on a selected gastric cancer cell line, HCG27. First, dose-effect curves for herceptin, irinotecan and 5-FU were created to serve as reference for the effectivity of herceptin. Later, Western blotting on several tumor markers was carried out to see the effects these three drugs had on HCG27. Results showed that, on gastric cancer cell line where HER2+ is overexpressed, herceptin had a clear anticancer effect.

INTRODUCTION

Gastric cancer refers to the presence of a tumor in any portion of the gastroesophageal junction to the pylorus. In 2016, there were 26.370 new human cases in the USA, representing a 20% increase over the last 5 years. **Adenocarcinoma** remains the predominant type of gastric cancer accounting for 95% of all solid stomach malignancies[1].

In the 1930s, gastric cancer accounted for around 30% of cancer deaths. Fortunately, due to a variety of reasons, like the lower prevalence of *Helicobacter pylori* infections due to improved health habits, and the change in the diet to include more fruits and vegetables, its mortality rate has dramatically decreased since then. In 2012, stomach cancer only accounted for 2% of total cancer deaths[2].

Still, even now the average 5-year survival rate for cancer is around 69%, and despite the improvements made in diagnosis techniques and chemotherapy, the 5-year survival rate for gastric cancer remains at 30%. Moreover, since the decrease in mortality was mostly due to an improvement of health and food habits, its incidence remains much higher in poor communities showing the highest disparity between white and black men, with a 2.5 times higher incidence in black men[2].

Worldwide, its incidence also differs, remaining highest in East Asia, whose cases represent 60% of new gastric cancer worldwide. In Japan, stomach and colorectal cancer remain the most common types of cancer[3].

Traditionally, **standard first line therapy for gastric cancer was 5-fluorouracil** (5-FU) monotherapy[4]. 5-FU is an antimetabolite fluruopyrimidine analog of the nucleoside pyrimidine with antineoplastic activity. Its mechanism of action is as a nucleic acid synthesis inhibitor[5]. Nowadays, 5-FU is still used, but in combination with other drugs like **cisplatin** (most common combination chemotherapy for gastric cancer), a metallic (platinum) coordination compound that crosslinks with the purine bases on the DNA, interfering DNA repair mechanisms, causing DNA damage and subsequently inducing apoptosis in cancer cells[6]. It has been argued that **docetaxel**, a taxane that induces cell death through mitotic catastrophe, should also be considered as a third drug. Although the efficacy of this three-drug combination therapy has been demonstrated, the added side-effects impede its use in patients in advanced stages or with deteriorated health[7].

In patients with advanced gastric cancer(AGC), **irinotecan**, a semisynthetic derivative from the plant alkaloid campothecin that inhibits DNA topoisomerase-I, thereby interfering with cell division and DNA replication, is often used as a second line therapy in combination with cisplatin in patients that show a strong reaction to 5-FU. Research on its plausibility as a first-line treatment on patients with AGC is also being conducted[8].

Still, **present treatments fail to provide acceptable long-term survivability** and in many countries second line treatments are still not considered as a viable option due to the perceived fatal prognosis of patients in whom standard first line treatment has failed[4].

Possibly due to the higher incidence and even lower 5-year survival rate, much of the research being done on new second and third line therapies for gastric cancer is being carried out in eastern countries like China or Japan[4]. One such possibility is that the application of **herceptin**, a recombinant humanized monoclonal antibody that targets the extracellular domain IV of the HER2 protein and traditionally used to treat breast cancer, could potentially serve as a second or third line treatment for gastric cancer patients who show bad tolerance to traditional treatments.

Recent trials on Japanese patients suffering from AGC show an improvement in both the prognosis and overall survivability in those cases that were treated with a combination of herceptin and chemotherapy[10].

Due to this potential, **herceptin was chosen as the focus for this experiment**, with 5-FU and irinotecan serving as comparison for standard first and second line treatments.

The effects of these drugs were studied on the **HCG27 cell line**, a cell line derived from the metastatic lymph node of gastric cancer. HCG27 has been often used on experiments related to herceptin and is characterized for its fast growth and its high expression of HER2 protein[11].

In order to better understand the effects that herceptin, irinotecan and 5-FU could potentially have on gastric cancer cells, it was decided to study the variation that **tumor markers**, defined as "a molecule, a process or a substance that is altered quantitatively or qualitatively in precancerous or cancerous conditions, the alteration being detectable by an assay"[12], would suffer in the HCG27 cell line after treatment with these drugs. For this purpose, a negative control known not to be expressed in HCG27 was checked. This test was composed of **Cyclin 1**, which promotes cell cycle progression[13]**, protein kinase R-like endoplasmic reticulum kinase** (PERK), which increases cancer cell proliferation[14] and **protein kinase B** (p-AKT), which reduces cell survivability[15].

On a second test it was decided to check on two proteins with anticancer effects, which in many cancers appear to be diminished or to have suffered from some kind of mutation. **Tumor protein p53**, which under stress regulates the expression of target genes[16], and **p53-upregulated modulator of apoptosis** (PUMA), a critical mediator of p53-dependent and -independent apoptosis and one of the most potent killers in its group[17], were the two selected proteins.

Lastly, the third and fourth tests were on proteins suspected to be modified by the selected drugs and with known carcinogenic effects. These proteins were **Bcl-xL**, an inhibitor of apoptosis which plays a crucial role on cancer development and resistances[18]**, endothelial p70 S6 kinase 1**, part of the mTOR/p70S6K1 pathway, which is suspected to be involved in carcinogenesis, metastasis, and chemotherapeutic drug resistance[19], and **phosphor-p38α**, part of the p38α MAPK pathway, a key factor in colorectal cancer therapy and chemoresistance[20].

On a final note, while not as common in **dogs**, the prognosis is equally bad for them, with most patients not surpassing the 6 months-mark. **Herceptin offers a new treatment possibility**, barely explored in veterinary sciences[21].

As mentioned above, herceptin targets the HER-2, a receptor exclusive to human beings. However, a HER-2 homologue called DER-2 (dog epithelial growth factor) has been found. Compared to its human counterpart, there was an amino acid identity of 92% and a homology of 95%. Recent studies have also shown that the targeting of DER-2 with herceptin led to the growth inhibition of canine tumor cells. More research is needed on the matter, but it could offer numerous advantages not only in the treatment of gastric cancer, but also in its original intended use in humans for the treatment of canine mammary gland tumors, the most common tumor in bitches[21-24].

JUSTIFICATION AND OBJECTIVES

This project is born from the collaboration with the Department of Pharmacology of the University of Yamaguchi during my exchange period in Japan, and my own interests in furthering my studies in pharmacology as well as learning how to conduct research.

The project was planned in conjunction with Shuhei Enjoji, a researcher in the aforementioned department, taking into account the resources availability of the department and my own capabilities.

The end goal of this work is to develop the necessary skills to conduct a proper research and to develop a further understanding of the mechanism of action of anticancer drugs and the effects they have on cancer cells.

This project is embedded in a bigger one, being carried out at the time in that department, whose objective is to show evidence of the feasibility of using herceptin as second or third line treatment in gastric cancer, as well as finding ways of increasing its effectiveness.

MATERIAL AND METHODS

Materials

Anticancer drugs were as follows: 5-FU (5-FU) (WAKO, Tokyo, Japan); irinotecan (LC Laboratories, Woburn, MA, USA), and herceptin (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan). Antibody sources were as follows: phospho-ERK p42/p44 (#9101), phospho-p38 MAPK Thr180/182 (#9216) phospho-Ser473 Akt1 (#07-310), Cyclin D1 (#2922), p53 (#9282), Bcl-xL (#2762), Phospho-p70 S6 Kinase (Thr389) (#9205), Puma (#4976), all from Cell Signaling Technology, Danvers, MA, U.S.A.). Secondary antibodies were as follows: Alexa Fluor 488 goat anti-mouse IgG; Alexa Fluor 488 goat anti-rabbit IgG; Alexa Fluor 568 goat anti-rabbit IgG; Alexa Fluor 488 donkey anti-goat IgG (Invitrogen). All other chemicals were purchased from Wako Pharmaceuticals (Osaka, Japan) [25].

Cell passage

The work is realized in a sterile environment inside of Class II Biosafety Cabinets. All pipettes and materials used are either sterile or have been properly disinfected.

The selected cell growth medium was DMEM (Dulbecco's Modified Eagle Medium), high glucose supplemented with 10% FBS and 1× antibiotic-antimycotic solution (Life Technologies Gibco, Carlsbad, CA, U.S.A.).

For cell passage, after vacuuming the existing DMEM, 0.5 ml trypsin/EDTA 1x was added twice, first to remove any remaining DMEM, and the second time in order to separate the cells from the petri dish, a process known as trypsinisation. The cells were then collected in a plastic tube and thrice as much DMEM was added, as trypsin can

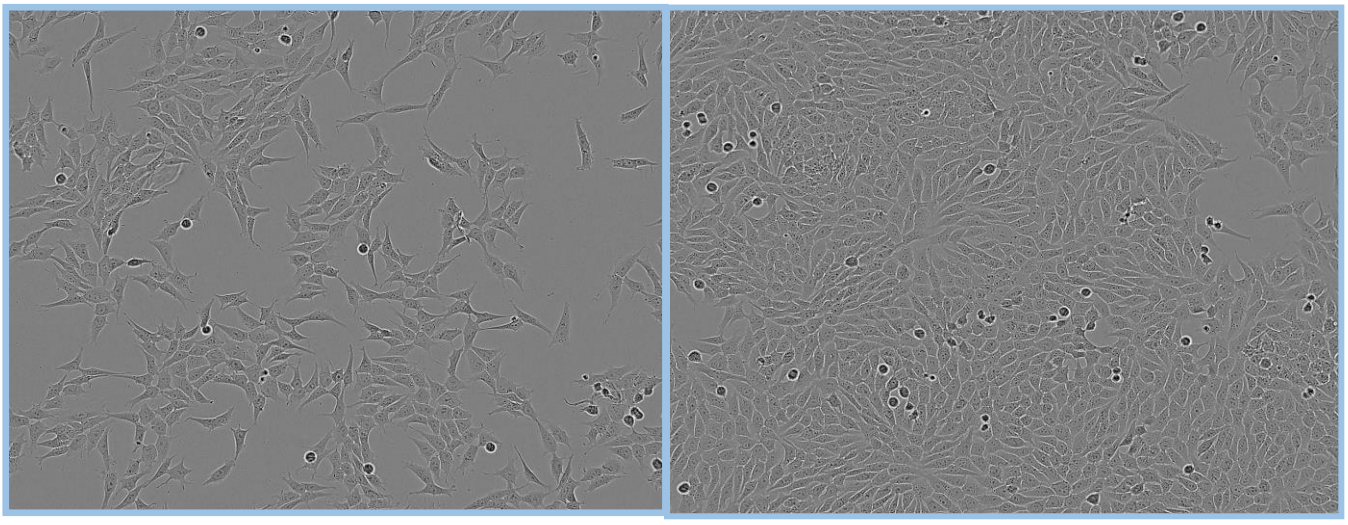

Figure 1 The picture to the left depicts HCG27 growth after 24 hours. The right one shows HCG27 growth after 48 hours

be toxic for cells after long exposures. Finally, 1/6th of this mixture was added to a new petri dish, and then 2-3 ml of DMEM were added.

Cell counting

After the cells were collected in the plastic tube and DMEM had been added, a Neubauer chamber was used to count the cells and find out their density.

Curve preparation

For the dose-effect curve, a flat-bottom, sterile, 24 wells, culture plate was prepared, and then each well was seeded with 5.0×10^3 cells/cm^2. 0.5 ml of DMEM per well was added and then the cells were carefully and evenly distributed across the surface of the well, to avoid higher concentrations around the center as it could lead to a diminished drug effect

Drug treatment

Cells attached after 6 hours, and treatment was applied after 24 hours. 5-FU and herceptin were prepared from 1 mg/ml drug stock solutions, while irinotecan was prepared from a 1mM stock solution. For each drug, 8 wells (4 pairs) were used.

5-FU	**1ml medium**	**Using 0.5ml medium**
1st pair	No treatment	No treatment
2nd pair	1 µg/ml	0.5 µg/ml
3rd Pair	5 µg/ml	2.5 µg/ml
4th Pair	10 µg/ml	5 µg/ml

Herceptin	1ml medium	Using 0.5ml medium
1st pair	No treatment	No treatment
2nd pair	1 µg/ml	0.5 µg/ml
3rd Pair	5 µg/ml	2.5 µg/ml
4th Pair	10 µg/ml	5 µg/ml

Irinotecan	1ml medium	Using 0.5ml medium
1st pair	No treatment	No treatment
2nd pair	0.586 µg/ml	0.293 µg/ml
3rd Pair	2.93 µg/ml	1.456 µg/ml
4th Pair	5.86 µg/ml	2.93 µg/ml

Drug concentrations were decided in accordance to previous experiences in the pharmacology laboratory in the University of Yamaguchi, and a study of related literature [26-31].

Cell survival

After a 48 hours incubation, the CCK-8 (Cell Counting Kit-8 by Dojindo) was used to check the survival of the treated cells. This kit is based on a reaction which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier.

It was applied on the cells after mixing with CGM and after an hour wait the solution was added to a 96 well plate and the fluorescence was checked in a Beckman Coulter fluorescence multimode detector DTX 880.

Western blot Protocol

Sample Lysis (from cell culture)

First, cells were washed in the culture dish by adding ice-cold HBS (Hanks balanced salt medium) and rocking gently. Then, PBS was discarded and lysis buffer was added. The amount of lysis buffer added depends on cell density. On this experiment, we used 100 µl/well for a cell density of 5.0 x 10^4 cells/cm^2. It is important to use the right amount of lysis buffer as high concentrations might excessively dilute the protein sample. Adherent cells were then scraped off using a plastic cell scraper, collected in 1.5 ml tubes and placed on ice.

After a 5 minute incubation on ice, an ultrasonic homogenizer was used for 5 seconds in each tube. Later, the cells were centrifuged for 15 min at 15.000 rpm. Finally, the supernatant or protein mix is collected in new 1.5 ml tubes, and the protein concentration was measured using Bio-Rad's DC Protein Assay, a colorimetric assay based on an improved version of the well-known Lowry-assay, greatly reducing the waiting times.

Sample Preparation

Knowing the concentration of protein in the extract was necessary for adjusting the amount of protein that is being loaded into each well, and ensure that it was consistent across all samples.

Later, samples whose protein concentration was then known were collected into new 1.5 ml tubes, and ¼ part of 5x SDS sample buffer were added. Amongst other components, this sample buffer contained glycerol, so that the samples could sink more easily into the wells of the gel, and bromophenol blue, a tracking dye that allowed us to see how far the separation had progressed.

The samples are then heated on a dry plate for 5 min at 100 °C in order to denature the higher order structure, which will enable the protein to move in an electric field. After denaturing, the proteins have a negative charge, and so, when a voltage is applied they will travel towards the positive electrode.

Gel electrophoresis

Two different kinds of polyacrylamide gels were prepared. Stacking gel, placed on top, is a porous gel with lower acrylamide content which separates protein poorly but allows them to form thin, sharply defined bands. And running gel, placed lower, has a higher acrylamide content and although porous, its pores are narrower, thus

allowing proteins to be separated by their size, as the smaller proteins will travel more easily and thus rapidly than larger ones. For this experiment, the gels had a thickness of 1.5 mm.

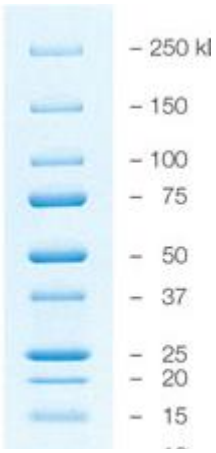

Figure 2 Protein ladder

Then, equal amounts of protein (10-30 µg proteins were used on different repetitions of the experiment) were loaded into each well, except for the one furthest to the left and the one furthest to the right. In the leftmost well, 3 µg of Bio-rad's Precision Plus protein Standards All Blue, a protein ladder with a range between 10-250 kD, was loaded, and on the rightmost well 1.5 µg of the same protein ladder was also loaded.

Next, a low voltage (80 V, 500 mA) is applied for 20 minutes, and then a higher voltage (120 V, 500 mA) was used until the blue marker of the sample buffer could be seen at the bottom of the gel, which was around 70 minutes on all instances.

Electrotransfer

After the protein mixture had been separated, it was transferred to a Bio-rad's Immun-blot PVDF membrane. This transfer was done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel, and into the membrane. A "sandwich" is prepared, placing a 4 cm x 10 cm fiber pad at each end, the membrane and the gel, both 4 cm x 10 cm are placed in the middle, protected on top and bottom by filter papers. To ensure a clear image, all parts of the sandwich were soaked in transfer buffer, and there must be a close contact between the gel and the membrane before starting the transfer. Transfer time depends on the thickness of the membrane. For this experiments 40 mA was applied for 100 minutes. Then the membrane can be cut depending on the different proteins that wish to be detected.

Washing, blocking and antibody incubation

In the next step, before antibodies could be added, blocking was necessary, in order to prevent antibodies from nonspecifically binding to the membrane. Blocking was done with 5 % skim milk in TBS-T at 4 ^{o}C for one hour.

Then, the membrane was washed for 10 minutes thrice with TBS-T to remove any remaining skim milk, and the primary antibodies, diluted to 1/1000 in TBS-T were added, and left incubating overnight at 4 ^{o}C.

After incubation, the membrane was again washed for 10 minutes thrice with TBS-T to remove unbound primary antibodies, and then the secondary antibodies were added. After 1 hour incubation, it was washed in the same

way, and then Amersham's ECL (enhanced chemiluminiscence) kit was applied over the whole surface of the membrane. Then, after a 1-2 minutes incubation the results could be visualized. In this experiment the Fujifilm LAS3000, based on CCD imaging was used for this purpose.

RESULTS AND DISCUSSION

DOSE-RESPONSE CURVE

As mentioned in the introduction, while the main focus of this experiment is herceptin, in order to properly understand how effective, or ineffective, it is, a comparison is needed. For this purpose, 5-FU, the standard first-line treatment, and irinotecan, one of the standard second-line treatments were selected.

For each curve, cell survival was checked three times, with two sets in each repeat. The average of these sets is presented in the following curves

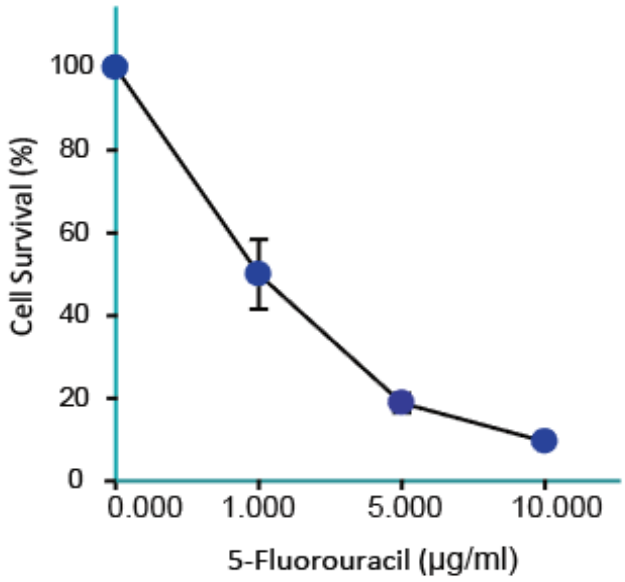

Figure 3 Dose-effect curve measuring cell survival at concentrations of 5-FU of 0.000 µg/ml, 1.000 µg/ml,, 5.000 µg/ml, and 10.000 µg/ml.

5-FU proved to be highly effective across all repetitions.

While on lower concentrations it showed a certain degree of variation, on higher concentrations, cell survival stayed consistently low with almost null deviation, as would be expected from the standard treatment for gastric cancer.

Irinotecan also showed to be quite variable on lower concentrations, and while this variation did remain on a much lower scale in mid concentrations, on the highest concentration, as was the case with 5-FU, cell survival remained consistently low

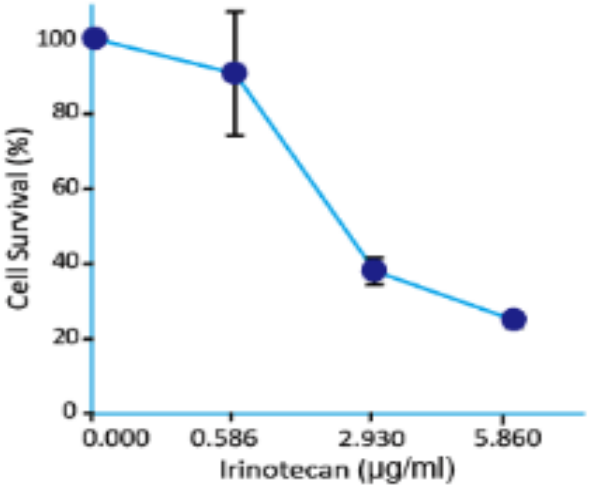

Figure 4 Dose-effect curve measuring cell survival at concentrations of irinotecan of 0.000 µg/ml,, 0.586 µg/ml,, 2.930 µg/ml, and 5.860 µg/ml.

For irinotecan a lower concentration was used compared to the other two drugs, as literature and previous experience in the pharmacology laboratory in the University of Yamaguchi show its effective dose to be lower[30].

Herceptin results showed a clear anticancer effect on HCG27 cancer cells, which corresponds with past affirmations of HER2 overexpression in this cell line [11].

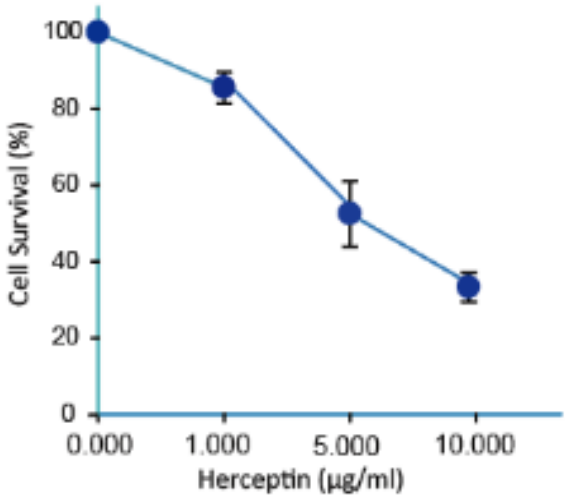

Figure 5 Dose-effect curve measuring cell survival at concentrations of herceptin of 0.000 µg/ml,, 1.000 µg/ml, 5.000 µg/ml, and 10.000 µg/ml.

However, despite the overexpression of HER2 in HCG27, compared to the results of the other two drugs, cell survival remained higher and more variable even on the highest concentrations. One possible explanation for these results, would be the existence of a resistance to herceptin in HCG27.

On breast cancer, instances of resistance in tumors where HER2 is overexpressed are not uncommon. For example, one study linked this resistance to the presence of the transmission factor STAT3, and after applying a STAT3 inhibitor treatment the effectiveness of herceptin was clearly increased[32]. Many other factors are involved in the resistance to herceptin and not all of them are well known.

In particular, one of the suspected resistance factors in gastric cancer are SET proteins, which modulate growth control and are overexpressed in gastric cancer. In the laboratory where this experiment was carried out, the planned next step of their experiments is to verify if there is any relation between the overexpression of these proteins and that of herceptin and to see if the effectiveness of herceptin is increased after the SET proteins are knocked down.

On the one hand, these dose-response curves allowed us to clearly compare the effectiveness of the three selected drugs. On the other hand, it served as basis for the Western blot, the second phase of the experiment.

As previously mentioned, the objective of Western blotting was to study the variation of different sets of tumor markers in the HCG27 cell line, but in order to obtain clear results there were two prerequisites. First, drug concentration and incubation time had to be enough for the drugs to have a clear effect on the cells, and second, a high enough number of cells had to survive so that sufficient protein could be collected. It was for this reason that the dose-effect curves were necessary.

WESTERN BLOT

While the dose-effect curves provided a valuable estimation on the incubation time and drug concentration, some trial and error was still needed. First, concentrations of 1.000 μg/ml, 2.500 μg/ml and 5.000 μg/ml for herceptin and 5-FU, and 0.586 μg/ml, 1.465 μg/ml, 2.930 μg/ml for irinotecan were used with a 6-hour incubation period. Cell survival remained close to 100% on all instances, so it was decided to increase incubation time to 12 hours. Although cell survival dropped, cells cultivated on the lowest concentrations still showed cell survival rates close to 100%. Therefore it was decided to increase the maximum concentration and forego the lowest one. For the final experiments, concentrations of 2.500 μg/ml, 5.000 μg/ml and 10.000 μg/ml for herceptin and 5-FU, and 1.465 μg/ml, 2.930 μg/ml and 5.860 μg/ml for irinotecan were selected. Incubation was kept at 12 hours.

Four different runs of Western blot were done. The first run, designed as practice in order to properly understand the technique, was focused on proteins known not to express in HCG27 and VCP/p97 (Valosin-containing protein), an easily detected protein used as a reference marker across all runs of Western blot in this experiment. While not recorded, the results of this run were negative, except for VCP/p97, which on the context of this experiment was a success.

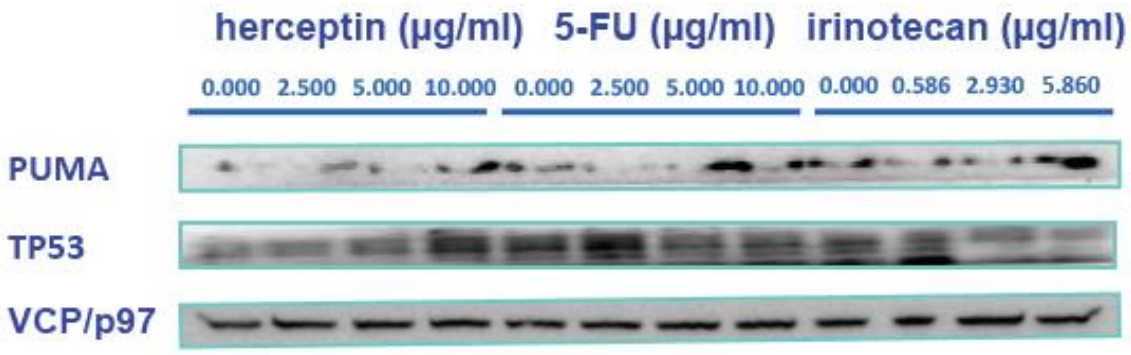

Figure 6 Results for the second run of Western blot, with 10% gel, 30 μg protein and a treatment time of 12 hours. 1x10^5 cells/well were seeded on a 12 well plate.

A bottleneck faced in this experiment was the limited availability of antibodies in the laboratory. For that reason, in the second run, the checked proteins were not specifically linked with HCG27, but with cell cancer lines in general. Starting from the bottom, VCP/p97 results came out clear and consistent across all lanes, a good indicator that the technique was correctly done.

Next is p53, a powerful cancer suppressor found either absent or mutated in most cancers. Mutant p53 proteins not only lose their tumor suppressive activities but often gain additional oncogenic functions increasing the survivability of cancer cells. For this reason, p53 Western blot results are often inconclusive, as an increase of a

mutant variation would be detrimental[16, 33]. In the results, p53 behavior remained inconsistent, as it increased on those cells treated with herceptin, but decreased on those treated with both irinotecan and 5-FU.

In order to better understand these results, we must look at the last protein of this run, PUMA. PUMA is a potent killer with several significant anticancer roles. It is regulated by p53, so in cases where p53 is mutant or absent, PUMA will always be absent as well[17]. While results were far from clear, we were able to see a marked increase on all 3 drugs. On the control lanes it appeared to be absent, but especially in the samples that were treated with the highest concentrations of the drugs, it can clearly be seen, even more so on those treated with irinotecan and 5-FU. This is important because it shows that all three drugs had increased normal p53 regulation, thereby improving tumor suppressing capabilities.

For the third and fourth runs, only proteins known to express in HCG27 and with interactions with at least one of the three drugs were selected.

Protein p38 was not added to the third run, as at the time it was done we lacked evidence of its expression in HCG27. Nevertheless, as both runs focused on the same proteins, both results shall be discussed together.

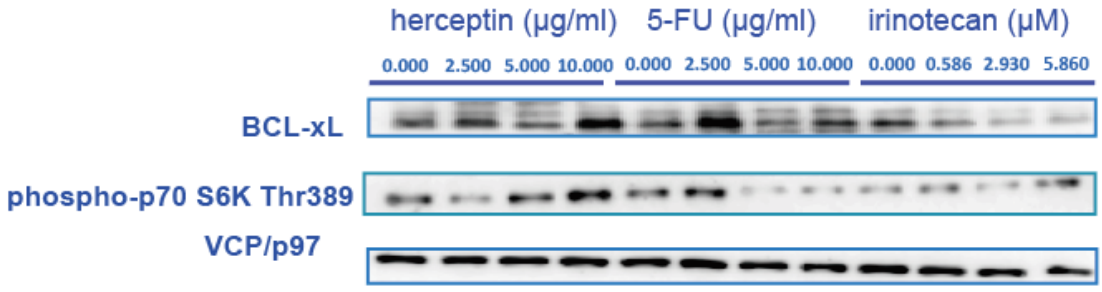

Figure 7 Results for the third run of Western blot, with 10% gel, 20 µg protein and a treatment time of 12 hours. 1x10^5 cells/well were seeded on a 12 well plate.

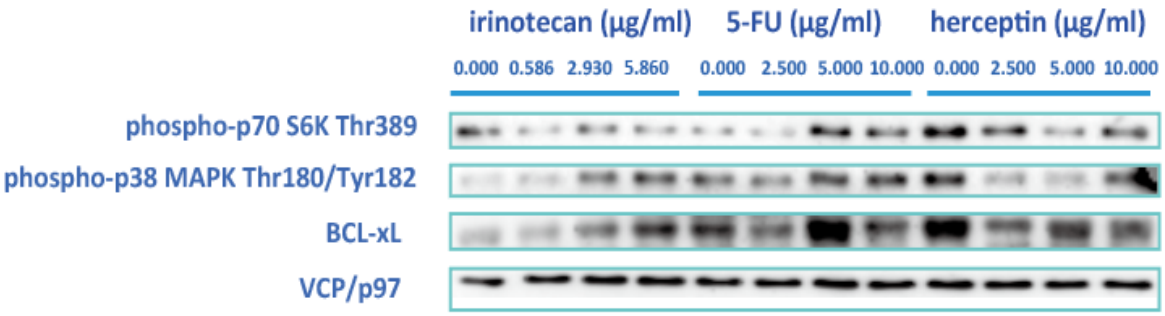

Figure 8 Results for the fourth run of Western blot, with 10% gel, 10 µg protein and a treatment time of 12 hours. 1x10^5 cells/well were seeded on a 12 well plate.

Before any further discussion, it must be established that these results are not enough to properly establish any correlation between the drugs and the effects they have on proteins. In order to do that, several repetitions with the same set of variables would need to be done. However, due to time constraints and the resources of this work, it was decided to limit the experiment to four runs.

However, and while the results are by no means definitive, it is still worth discussing, as several initial conclusions may be drawn that could help us elucidate what approach to follow should this experiment be continued in the future.

First, we will focus on the phospho-p38 MAPK protein, whose interest resides in its participation in the p38α pathway. This pathway has been shown to have a dual nature, as under normal circumstances it serves as a negative regulator of proliferation even in cancer cells. However, in many instances the proteins proliferate out of control, and the pathway acquires an oncogenic role involving several cancer related-processes such as invasion, inflammation and angiogenesis. Moreover, resistance to irinotecan and 5-FU in colorectal cancer appears to be linked to this particular pathway[20]. In our results, it appeared to have an erratic behavior, appearing to increase at higher concentrations of irinotecan and 5-FU and to decrease in those cells treated with herceptin. A possible explanation would be the aforementioned resistance that this protein shows against irinotecan and 5-FU. Herceptin does not appear to be influenced by this resistance, which would make it a suitable alternative in cases where this pathway is active.

Next is BCL-xl, which in many instances of colorectal cancer is the only anti-apoptotic protein being overexpressed in the malignant state, and whose inhibition has been shown to decrease carcinogenesis and improve anti-cancer therapy, as this family of proteins also induce drug resistance in cancer cells [18, 34]. Results were conflicting, which would call for more assays, as while in the third run it seemed to either increase or remain constant in cells treated with herceptin, it appeared to decrease in the fourth run. Irinotecan had a similar pattern as Bcl-xL levels seemed to increase in the fourth run, but appeared to decrease in the third run.

A possible explanation might be found in the amount of proteins loaded in the wells, this being the only difference amongst these runs that due to sample availability was decreased in the fourth run, and might affect how these drugs behaved. Finally, in those cells treated with 5-FU, Bcl-xL levels peaked with low-to-mid concentrations in both cases, decreasing again at higher concentrations. This could indicate that the effective range of 5-FU for Bcl-xL is rather limited, and if this was the chosen treatment, it would have to be carefully adjusted.

Endothelial p70 S6 kinase 1 activation is known to enhance cell-induced tumor growth and angiogenesis, although the exact mechanisms by which it does so are yet to be elucidated[19]. Results again appeared to be conflicting. Irinotecan did not seem to have had much effect and while 5-FU showed some change, the variation between the two runs make it difficult to draw any conclusion without further research. Lastly, while in the third run cells treated with herceptin also showed variable levels of endothelial p70 S6 kinase 1, on the fourth run it appeared to be decreasing, a promising result given the oncogenic actions of this protein.

As has been repeatedly stated, further research would be needed in order to actually stablish the viability of herceptin as a second or third-line treatment for gastric cancer. This would imply, not only repeating the last runs of Western blot to obtain more solid results, but also checking more tumor markers in several other gastric cancer cell lines as well as looking for ways to bypass the resistances found in some tumors which overexpress the HER2 factor and yet herceptin effectiveness in them is limited.

Nonetheless, as far as the scope of this experiment is concerned, herceptin showed an effect on all the checked tumor markers and cell lines treated with it showed, although not at the levels of 5-FU, a clear reduction in cell survivability, which proves the feasibility of it becoming an alternative to current gastric cancer treatments.

CONCLUSIONES

La herceptina tuvo claros efectos anticancerígenos, como se deduce de la notable disminución de la supervivencia celular que muestran las curvas dosis-respuesta realizadas sobre HCG27. Además, tras realizar Western blots de diversos marcadores tumorales, todos los marcadores habían sido modificados por la herceptina. Sin embargo, para poder demostrar si la herceptina puede llegar a ser un tratamiento de segunda o tercera línea viable serán necesarias más investigaciones. Dado el limitado número de tratamientos disponibles para el cáncer gástrico avanzado, herceptina tiene el potencial de ser una alternativa viable a los tratamientos actuales.

CONCLUSIONS

Herceptin showed clear anticancer effects, as evidenced by the marked decrease in cell survivability shown in the dose-response curves for HCG27. Furthermore, after Western blotting several tumor markers expressed in HCG27, all of them had been modified by the effects of herceptin. Although further research will be needed to demonstrate whether herceptin could become a viable second or third-line treatment, given the limited number of treatments available for advanced gastric cancer, herceptin has the potential to become an alternative to current treatments.

PERSONAL EVALUATION

The realization of this work allowed me to catch a glimpse of the world of biosanitary research, something that was of great interest to me. Furthermore, cancer research is currently one of the most interesting research topics in human and veterinary medicine, and being able to participate on the first stages of a possible new treatment was a tremendous opportunity.

The whole experiment was completed during my stay in Japan, which on the one hand allowed me to learn a different work way, but on the other hand presented several challenges such as time constraints or overcoming the language barrier.

All in all, it proved to be a great learning experience, and I feel really grateful for the help I received from all the other members of the laboratory during my stay, and the patience of Drs. Jesus Garcia and Jose Aramayona after coming back to Spain.

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APPENDIX

0.2 % SDS Lysis Buffer: refrigerated at -20°Clanes

Stock	Volume	Final concentration
1M Tris-HCl (pH 8.0)	5 ml	50 mM
0.5 M EDTA (pH 8.0)	1 ml	5 mM
0.5 M EGTA (pH 8.0)	1 ml	5 mM
10% SDS	2 ml	0.2%
Nonidet P-40 (IGEPAL CA-630)	500 µl	0.5%
Na3VO4 (Sodium Vanadate)	18.4 mg	1 mM
Sodium Pyrophosphate	532 mg	20 mM
Roche Complete	2 tablets	
Milli-Q Water	to 100 ml	

5X Sample Buffer: kept at room temperature

Stock	Volume	Final concentration
1M Tris-HCl (pH6.8)	2.5 ml	50 mM
10% SDS	2 ml	0.4%
Beta-mercaptoethanol (2ME)	3 ml	6%
Glycerol	1 ml	2%
Bromophenol Blue	As necessary	
Milli-Q Water	1.5 ml	

10X Running Buffer: refrigerated at 4°C

Stock	Volume
Glycine	144 g
Tris	30 g
SDS	10 g
Milli-Q Water	to 1 L

10X Transfer Buffer: refrigerated at 4°C

Stock	Volume	Final concentration
Glycine	112.6 g	150 mM
Tris	60.5 g	50 mM
Milli-Q Water		to 1 L

TBS-T: kept at room temperature

Stock	Volume	Final concentration
20X TBS	50 ml	
Tween 20	1 ml	0.05%
Milli-Q Water	950 ml	