In vivo effectiveness tests of the Cytoreg® formulation against melanoma B16F1 in female mice C57BL/6//BIOU

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Abstract

With the purpose of extending the pre-clinical studies of the formulation Cytoreg® (Cytorex International Inc., Maracaibo, State of Zulia, Venezuela) as an antineoplastic therapy alternative, in the present assay effectiveness of this formulation was evaluated on induced melanoma by cells of the B16F1 line, maintained in mice females C57BL/6//BIOU. In the experiment, 30 mice females were distributed of the following way: sick control (10 mice), sick group with treatment of Cytoreg® (10 mice), sick group with the conventional treatment with interferon α-2b (5 mice) and healthy control group (5 mice). All the experimental mice were inoculated with melanoma cells B16F1, except the healthy control group. Daily consumption of water and of food was evaluated, as well as the body weight and the development of the tumor. The hematologic parameters evaluated were: white leukocytes, hematocrits and differential count. The results demonstrate that formulation Cytoreg® stimulated the increase of the concentration of leukocytes, did not alter the percentages of the hematocrits, and increased of the neutrophils and eosinophils percentages. The tumor´s size in the mice treated with Cytoreg® formulations was similar to that of the conventional treatment mice.

Key words: Cancer, Cytoreg®, mouse *C57BL/6//BIOU*, melanoma.

Introduction

Cytoreg® is a chemical formulation developed by the Cytorex International, Inc. laboratory in the state of Zulia, Venezuela, in order to be used as an alternative chemotherapy against cancer cells. The incorporation in the market of a new drug contemplates several stages, one of them being the preclinical studies, with the purpose of evaluating the biological activity, the toxicity, the effect on the reproduction, the progeny and the pharmacokinetic properties, among others. These studies are carried out on animals, taking into account the results of a previous analysis of the physical-chemical properties and behavior of the compound in vitro (1).

In previous in vitro studies, the Cytoreg® presented a dose-growth time dependent effect, in addition to an inhibitory effect in all cells by the induction of apoptosis via CPP32, independent of the hormonal sensitivity of the cells. The data showed that not only could the CPP32 pathway be a potential target for the regulation of apoptosis induced by Cytoreg®, but could also play a significant role in the chemotherapeutic regimen in many malignant human tumors (2). Within the studies carried out with the formulation are the application on skin lesions caused by fungi (3); in addition, preclinical studies have been made: the determination of lethal dose 50 (LD50), assessment of acute and chronic toxicity, which were performed in females of the rat species of the non-consanguineous BIO line: Wistar, chronic toxicity in rabbits (4), in addition to pharmacokinetics.
In order to extend the preclinical studies with the Cytoreg®, the objective in this work was to evaluate the effectiveness of the formulation on melanoma caused by cells of line B16F1 (5), cultured in vivo in female mice C57BL/6//BIO (6), produced in the Vivarium of the University of Los Andes, Mérida-Venezuela.

Materials and methods

Biological material: We used 30 female mice of the C57BL/6//BIOU line, between 8 and 10 weeks of age, produced and maintained in the Vivarium of the University of the Andes (BIOULA), fed with commercial Ratarin (Protinal), treated with temperature 120 ° C for 1 minute and supplied at will, as well as water (sterilized at 120 ° C for 20 minutes). Groups of mice were housed in T2 boxes (26 x 21 x 24) cm, maintained at a temperature of 21 ± 4 ° C, and 12 light hours: 12 dark hours. The mice were distributed as shown in Table 1.

10 mice were used to test the effectiveness of the formulation, group identified as D1C; this number was selected due to the need to have a statistically significant number to ensure that the effects that would be observed could be attributed to the administration of the formulation, since the formulation under test is a first-generation product which is under evaluation with the intention of being taken to human

consumption. 10 mice were used with the tumor, a group identified as Sick Control, in order to have a similar number to the D1C group. The groups of mice used for the administration of the conventional therapy: Interferon α-2b (INT A), and the healthy control group, was 5 mice each. The same number of mice were not uses, as the other groups involved in the experience, because a conventional therapy that is already applied in the treatment of melanoma in humans must have been sufficiently validated; with regard to healthy mice, due to their own health condition, the use of a large number of mice was not considered, since these would be sentinels of the environment to check for the non-existence of factors.

Obtaining B16F1 melanoma cells

B16F1 murine melanoma cells were donated by Dr. Juan Luis Concepción, from the Parasite Enzymology Laboratory of the Faculty of Sciences of the Universidad de Los Andes - Mérida. These were extracted from a tumor from a male C57BL/6 mouse. After removing the tumor capsule, the cells were sucked through a syringe, and placed in PBS glycosylated at 0.9% (pH 7.4). Subsequently, a mechanical dissociation of the extracted cells in a petri dish was carried out in order to eliminate any remaining tissue, using syringes of decreasing caliber, continuously. The count of the cells to be inoculated was then carried out in a volume of 1 to 1 in PBS glycosylated at 0.9% (pH 7.4). Cell counting was performed in the Neubauer chamber.

Inoculation of cancer cells

Inoculation of the cells was performed intramuscularly in the left posterior thigh, in a volume less than 0.1 mL containing 5 x 10^5 murine melanoma cells, in 25 female mice randomly selected from the 30 expected to be used in the study. This route facilitated the effective replication, easy follow-up and measurement of tumor growth, which was evaluated during 10 post-inoculation days to, later, initiate the treatments.

Cytoreg® effectiveness tests

After 10 days post-inoculation of the melanoma cells, the treatments were administered: orally (intragastric involuntary) in a daily dose of 0.49 mL/Kg of weight (7) of the formulation Cytoreg® to a group of 10 mice; at the same time, the conventional treatment, INT A, was administered; this was administered by intraperitoneal injection, three times a week in a dose of 66 IU/kg dissolved in PBS pH 7.4 (8), to a group of 5 mice. To 10 of the remaining inoculated mice, none of the treatments was applied, the latter being the sick control group (CE). To study the effectiveness of the formulation, melanoma growth was followed, for which the diameter of the tumor was measured using a Vernier whose

measurements were recorded in centimeters; this measurement was performed twice a week, for 2 weeks. The treatments were administered for 15 days.

Monitoring of the physiological state of the animal

The consumption of water and food was evaluated daily, to all the animals of the study, during the treatment period. The animals were weighed, using an SF-400 scale (capacity 5000gx1g/200oz X 0.102), at the beginning, during and at the end of the treatment, and were performed hematological tests, for which blood samples were taken from the vein of the tail (9), at the beginning of the treatment and at the end of the treatment. The hematological tests performed were: hematocrit, leukocytes and differential count (neutrophils, lymphocytes, eosinophils, monocytes and basophils). In the hematocrit assessment, the blood was centrifuged in a micro-hematocrit Model *CriptSpin*. To perform the white blood cell count, the Neubauer chamber was used, diluting the sample in Turk's solution, and for the differential count a smear was performed, which was stained with 10% Giemsa (10). The total blood used for the tests was 3 drops, approximately 0.3 mL; samples were also taken from the healthy mice, in the same period of time.

Statistical tests.

As the intention was to determine if the formulation under test affected the growth of the tumor, the experimental design was carried out proposing 3 experimental groups that allowed to compare the development of the tumor: under the influence of a conventional treatment (INT A), one without treatment (CE) and the other with the treatment under test. The data were processed using the Kolmogorov-Smirnov test as an adjustment test to review the distribution of the data; the ANOVA statistical analysis was performed, considering it statistically significant for $p < 0.05$ and, subsequently, the T test for two independent samples was used (Two Sample T test), to perform the comparison of data between treatments

For the statistical analysis, the tumor growth results were compared between the mice that were treated (D1C and INT A) and the sick control (CE).

Although the aim of the work was to evaluate the effectiveness of the Cytoreg® formulation and, with the previous statistical analysis, the objective could be met, some physiological parameters were also evaluated, such as: water consumption, food consumption, body weight and hematological parameters. The tests for the statistical analysis of these results were the same as those used in the previous analysis: Kolmogorov-Smirnov as adjustment test to review the distribution of the data, ANOVA (one way AOV) and the T test for two independent samples (Two Sample T test); with the exception of the analysis of the body weight, in all the others the results of the four study groups were considered: CE (sick control), D1C

(treated with the test formulation), CS (healthy control) and INT A (with conventional treatment); statistically significant values for $p < 0.05$ were considered. All statistical tests were performed using the statistical software Statistix7.0 (11); this work was approved by the Bioterium Ethics Committee of the Universidad de Los Andes.

Results

The consumption of water and food of the animals that received the treatments and controls was fluctuating during the 15 days of registration. With respect to water consumption, the mice of the D1C group consumed an average of 2.4 mL/day /mouse; the mice of the CE and INT A groups, of 2.9 mL/day/mouse, in contrast to the CS group that had an average of 3.8 mL/day/mouse. In relation to the consumption of the food, the average daily consumption per mouse during the 15 days was 3.1 g for the D1C and 3.3 g for the mice subjected to the treatment with INT A. It was observed that the sick mice, without any treatment (CE), had the lowest average consumption of food, which was 2.0 g/day, and the average consumption of the healthy control group was 2.7 g.

The results obtained in the Kolmogorov-Smirnov statistical test led to reject the null hypothesis, Ho: the sample does not present a normal distribution, for both physiological parameters; when performing the ANOVA test, significant statistical differences were obtained for water consumption and for food consumption, with $p < 0.05$ respectively, leading to accept the Ho: water consumption is not equal between the groups involved in the study, and, Ho: the consumption of food is not equal between the groups involved in the study. The results of p of the comparison T tests of two samples are presented in Table 2.

Behavior of body weight

Before inoculating the melanoma cells to the groups under test, the weight of the animals was between 17.9 and 23.9 g. At the end of the experiment, the weight of the sick, untreated (CE) and treated mice (D1C e INT A), were found between 27 g and 29 g, respectively; for some of these mice, by ethical principles in the use of the laboratory animals of the Vivarium, the "end point" was applied; the euthanasia was applied to three mice of the control group and 2 mice of the group treated with INT A; for the latter, the excess weight was 7 g, probably corresponding to the weight of the tumor. None of the mice in these two groups exhibited normal behavior inside the box. The average weight of the group of healthy control mice was 23 g, and like the mice treated with the formulation under test, both showed a normal behavior inside the box. The results of the evaluation of the Kolmogorov-Smirnov statistical test, of the data obtained from weight versus time, of the D1C, INT A and CE groups led to reject the null hypothesis, Ho: the sample did not present a normal distribution ($p > 0.05$); when performing the ANOVA

test, weight versus treatment, significant statistical differences were obtained, in the analysis of the comparison of the groups by means of the T-test was obtained, between CE vs. D1C and CE vs. INT A, p <0.05 and for D1C vs INT A, there were no statistically significant differences. The results lead to approve the null hypothesis, Ho: the body weight of the group D1C is not

Behavior of melanoma tumor size

It could be observed with respect to the growth of the tumor that all the mice inoculated with the melanoma cells had a continuous growth in all the groups. When analyzing the data (size vs. time) using the Kolmogorov-Smirnov test, it was found that there were no statistically significant differences, so the null hypothesis is rejected, Ho: the sample does not present a normal distribution, indicating the existence of a normal distribution of the data, so these were analyzed by means of a one-way analysis of variance (one-way AOV). For the results found in the comparison of the groups by means of the T test, it was found for CE vs D1C and CE vs INT A, statistically significant differences and for INT A vs D1C, not significant, p> 0.05.

Hematological parameters

In Table 3, the averages of the values found for the hematological parameters can be observed: leukocytes and hematocrit evaluated, before the administration of the treatments and at 15 days post treatment. In the statistical analysis, significant differences were found.

In Table 4, the averages of the values found for the differential count of the cells can be observed: neutrophils, lymphocytes, eosinophils, basophils and monocytes, before the administration of the treatments and at 15 days post treatment.

Statistical analysis, using the Kolmogorov-Smirnov test, showed values of p> 0.05 for all parameters evaluated, indicating a normal distribution in relation to time: initial (t0) and final (t15 days). In the ANOVA performed, data versus treatment, no significant differences were found, except for the D1C and CE groups for the basophilic and monocyte parameters, and the CE group for the percentage of neutrophils, for p> 0.05; and when comparing the groups of treatments by means of the T test at the final time, statistical differences were found between the groups: D1C vs EC for the parameters white blood cells (p = 0.0127 *) and neutrophils (p = 0.0026 *); D1C vs CS for the eosinophilic parameter (p = 0.0135 *); D1C vs INT A for the neutrophil parameters (p = 0.0108 *); lymphocytes (p = 0.0009 *) and eosinophils (p = 0.0040 *); CS vs EC for neutrophils (p = 0.0162 *); INT A vs CS for neutrophils (p = 0.0321 *); lymphocytes (p = 0.0009 *) and eosinophils (p = 0.0040 *); INT A vs EC for white blood cells (p = 0.0007 *).

Discussion

In this work, the melanoma tumor growth was evaluated along with some physiological parameters, in order to evaluate the effectiveness of the Cytoreg® formulation. The results obtained allowed to determine the efficiency of this with respect to the tumor growth retardation and the response physiological treatment of mice treated for 15 days.

The evaluation of physiological parameters, in general, allows to assess the health status of animals that are subjected to various tests in laboratories. The deviation from the normal values of such parameters lead to suspect any adverse effects of the tests. According to Arencibia et al. (12), the normal water consumption of animals is 10% of the live weight. The water consumption by the mice of the healthy control group was 3.8 mL, being higher than that proposed by the aforementioned authors, since the average body weight observed was 23 g; based on this value, it was observed that no significant statistical differences were obtained between the healthy control group (CS) and the treated groups, and statistically significant differences were observed between these and the control group, considering that the pathology without treatment affects the consumption of Water.

According to Hernández (13), the food consumption of mice is 1.3 to 1.5 g per 10 g of body weight per day. For the mice of the healthy control group, the average weight was 23 g, and that of the feed intake was 3.2 g/10 g of weight, observing a relationship with that proposed by the author. This behavior was not observed by the mice of the rest of the groups (D1C, CE, INT A), during the 15 days of treatment, which presented significant differences ($p < 0.05$). The food consumption of the mice with the pathology (CE, INT A and D1C), did not show a relationship between the increase in weight and the consumption of the food; according to the author's proposal, they consumed less food; the average weight of the mice with the pathology was 26 g and the average food consumption was 1.2 g of food, therefore, it is considered that the presence of the tumor affected the consumption of food.

Regarding body weight, significant differences were observed between groups D1C, CE INT A ($p = 0.0000$), which could be related to the size of the tumor, since when comparing this parameter between these groups. However, no statistically significant differences were observed between the weight reached by the mice of the INT A and D1C group, but statistically significant differences were found between these two and the sick control group. The tumor measurements during the 15 days of treatment in the mice of the three groups under test, by means of the ANOVA statistical analysis, did not present significant differences between the groups of treated mice: INT A and D1C, with $p = 0.3095$; however, significant differences were observed between the treated groups and the sick control. The conventional treatment INT A, is a cytokine-based therapy that is often used against metastatic melanoma in humans

(14); its effect in conjunction with other antineoplastic drugs such as diosmin (15), in pulmonary metastatic melanoma (16, 17), ; in this study, the average tumor size in the animals treated with INT A, had the same growth as that of the animals treated with the Cytoreg® formulation; mechanisms such as the induction of apoptosis of the cancer cells (18), or the interference of the tumor signaling pathways that cause lung metastasis of melanoma B16F1 (19), could be involved in the effect of Cytoreg® on melanoma tumor development; however, specialized or deeper studies should be carried out to verify the mechanisms by which the growth of the tumor is delayed with respect to the Sick Controls, when the animals are treated with the formulation. In the analysis of hematological parameters, it must be taken into account that leukocytes, in pathologies such as cancer or in the use of certain drugs, the relative and absolute number of the different types often reflect the type, duration and severity of the illness. A variety of factors, including bacterial infections, cancer, stress and chemotherapy, cause the increase or decrease in the number of neutrophils, a type of leukocyte.

In the experience, it was found that in the statistical analysis (T test) for the values of the hematological parameters, before starting the treatment and after 15 days of treatment, only the values of the percentage of basophils and monocytes for the mice of the groups CE and D1C presented statistically significant differences ($p < 0.05$), indicating that there are differences between the values before and after treatment; the same behavior was observed for the percentage of neutrophils but in the D1C group; the rest of the evaluated parameters did not present statistical differences when the initial values were evaluated with respect to the final values.

In the bibliography it is reported as normal that the percentage of lymphocytes is higher than that of neutrophils in mice (20); this behavior could be observed for the groups involved in the study, with the exception of the D1C group, although the leukocyte concentration was found within the range considered normal for the mice.

Neutropenia (decrease in neutrophils) is the most frequent hematological adverse effect in patients with cancer; the Cytoreg® formulation, diverts the lymphocyte to neutrophil ratio considered normal for mice to neutrophils to lymphocytes. According to the literature, the formulation would act as an immunomodulatory, influencing the differentiation of hematopoietic cells (21, 22).

In general, monocytopenia is a condition that affects individuals when undergoing chemotherapy (23); the results obtained indicate that the mice that were subjected to the consumption of the formulation under test presented a condition of monocytosis instead of a monocytopenia, which indicates a recovery from neutropenia, characteristic of oncological pathologies. It was also possible to observe an increase in the basophils (basophilia) in the mice that received the formulation; none of these two conditions were

observed in the mice subjected to the conventional treatment INT A, who presented a condition of monocytopenia and basophilia, both characteristics of the pathology suffered (24), which was present in the mice of the sick control group. The EC mice and those treated with Cytoreg presented eosinophilia; this finding characterizes the inflammatory pathologies (25, 26).

In the mice of the healthy control group, the hematological values remained without significant differences throughout the experience; this result was corroborated when no significant differences were observed for a $p = 0.0918$.

Conclusions

It was concluded that Cytoreg® exerts a positive effect in the increase in the concentration of white blood cells and in the percentage of neutrophils, eosinophils, monocytes and basophils, and in the non-alteration of the hematocrit, a condition that must be present in mice when there is health in them, managing to overcome certain pathologies. The Cytoreg® formulation presented what could be considered a similar effectiveness, regarding the development of the tumor, to the one presented by the conventional therapy INT A, which, although it did not stop the growth of the tumor in 100%, it was less than that presented by mice that were not treated.

Table 1. Distribution of the number of animals of each of the experimental groups.

Grupo	Experimental groups	No. of animals	No. of animals per T2 box
1	Sick Control (CE)	10	5
2	Mice treated with formulation Cytoreg® (D1C)	10	
3	Mice treated with conventional treatment Interferon α-2b (INT A)	5	
4	Healthy Control (CS)	5	

Table 2. Value of p for the relation of water and food consumption comparing treatments, by means of the T-test of comparison of two samples.

	D1C vs CE	D1C vs CS	D1C vs INT A	INT A vs CE	INT A vs CS	CE vs CS
Water consumption	0.1064	0.0000 *	0.0063	0.6346	0.0003 *	0.0001 *
Food consumption	0.0198 *	0.3713	0.0596	0.0458 *	0.0758	0.0401 *

*Statistically significant

Table 3. Average values of leukocytes and hematocrit obtained in the different groups of treatments and controls.

Treatment Group	leucocytes (cell/mm3) t_0	leucocytes (cell/mm 3) t_{15}	Hematocrit (%) t_0	Hematocrit (%) t_{15}
CE	7.515±1166.3	6.2107±1166.3	45.4±3.4	30.1±3.0
D1C	11.825±2715.2	14.145±2715.2	50.1±2.8	39.8±4.6
INT A	12.670±12730	10.550±5770.2	47.5±4.8	35.6±2.2
CS	13.500±13480	13.400±3955.0	56.0±3.7	55.3±2.0

CE: Sick Control. D1C: Mice treated with formulation Cytoreg® INT A: Mice treated with conventional treatment Interferon α-2b (INT A). CS: Healthy Control. (± SD).

Table 4. Average values of the differential count obtained in the different treatment groups and the controls before (t_0) and at 15 (t_{15}) days of treatment.

Grupos	CE	D1C	INT A	CS
Neu (t_0)	36.8±0.2	30.0±2.1	29.4±0.8	21.1±0.3
Neu (t_{15})	61.1±1.1	35.6±0.8	62.2±0.7	16.7±1.2
Lin (t_0)	63.5±1.2	79.6±0.8	68.2±1.5	78.6±2.2
Lin (t_{15})	58.3±1.1	58.3±1.7	54.4±2.1	74.6±1.6
Mon (t_0)	0.7±1.1	1.6±0.9	0.4±1.4	0.4±1.3
Mon (t_{15})	2.3±1.1	0.5±0.6	0.6±0.8	0.4±0.9
Eos (t_0)	2.2±0.4	4.4±1.2	1.4±1.2	0.0±0.5
Eos (t_{15})	4.3±0.3	5.0±2.1	2.6±1.1	0.4±0.2
Bas (t_0)	1.1±0.9	0.5±0.9	0.0±0.6	0.0±0.5
Bas (t_{15})	1.4±0.6	0.2±0.4	0.0±0.5	0.0±0.2

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