

REPORT N° CD-92/3089F

ANTITUMORAL ACTIVITY.

LEWIS LUNG CARCINOMA IN MOUSE B6D2F1

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Place of Study : RESEARCH AND APPLIED DEVELOPMENT
CENTER S.A.L.
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This report has been done following the norms of Good Laboratory Practice published by the OCDE (1981). No circumstances that can affect the fiability of tha data have been registered.

General Director:

N. Basi

July 12th, 1993.

Pharmacodinamia area Dtr.

M. A. Arañó

July 12th, 1993.

Study Director

M.I. Zapatero

July 12th, 1993.

UNITY OF QUALITY WARRANTY. (UQW)

Inspection of the report n° CD-92/3089F

Following the regulations of "Good Laboratory Practice" of the OCDE, this study has been inspected and its report checked joint with the Normalized Work Procedures of UGC.

The dates in which the inspections were made are detailed below.

Date	Phase	N° Inspection UGC	Rep. to Dir.
Sep.10, 92	Protocol	6037	Sep. 10, 92
Apr. 14, 93	Lewis Lung cells Inoculation, via i.m	6968	Apr. 15, 93
Apr. 28, 93	Wheighing, sacrifice and obtention of tumo ral cells	7066	Apr. 28, 93
May 10, 93	Inoculation i.m. to mouses B6D2F1	7122	May 10, 93
May 11, 93	Wheighing and admon i.p.	7135	May 11, 93
May 18, 93	Wheighing and admon i.p.	7166	May 19, 93
May 31, 93	Sacrifice and extrac tion of primary tumor lung and recounting of metastasis	7232	Jun. 1, 93
May 31, 93	Wheighing of the pri mary tumor and lung	7234	Jun. 1, 93
July 5, 93	Final report	7397	Jul. 8, 93

A. Flores (signed)
Unity of Quality Warranty

July, 12th, 1993.

Summary.

The antitumoral activity of the substance FR-91 has been evaluated at three levels of doses in the experimental model of Lewis Lung Carcinoma in the mouse.

The substance has been administrated at the doses of 0.1, 0.3 and 0.5 ml/animal intraperitoneal via for 11 days.

As a substance of reference CICLOPHOSPHAMIDE has been used at a dose of 20 mg/kg intraperitoneal via for 11 days.

The Control group has been administrated physiologic serum.

The administration of the substance of reference, CICLOPHOSPHAMIDE produced an inhibition of the weight of the primary tumor of 15.4% and an inhibition of the weight of the lung metastasis of 97.6% in relation with the Control group.

The administration of the essayed substance FR-91, did not inhibit the weight of the primary tumor, but inhibitions of the weight of the lung metastasis of 8.2, 24.0 and 35.9% in relation with the the Control group for the doses of 0.1, 0.3 and 0.5 ml/animal respectively, were observed.

In our experimental conditions, the administration of the substance FR-91 has not shown antitumoral activity, although a slight inhibition of the lung metastasis has been produced.

1.- Objective.

The objective of this study has been to valorate the antitumoral activity of the FR-91 substance at the experimental model of Lewis Lung carcinoma in the mouse.

2.-Justification.

The lympholeukemia L1210 and Lymphocitic leukemia P388 are the essays of election to select antitumoral pharmacos of sintetic and natural origin.

Based on the studies made about the cinetic of tumoral cells growing, the National Institute of Cancer suggested the utilization of the Model of Lewis Lung Carcinoma, since being this a slow growing tumor, allows to discover products that can distroy tumors with a relatively high percentage of non proliferative but viable cells.

Lewis Lung carcinoma in the mouse appeared spontaneously in 1951 as a lung carcinoma in the mouse C57BL/6. It is a solid tumor with a cicle of 21 days.

After 6-7 days of inoculation a primary tumor is already seen;this can reach a very big size. From this lung metastasis appear and they are easily identificable and quantifiable.

Mouses C57BL/6 for keeping the tumor and mouses B6D2F1 to make the essay the way the protocol of the National Institute of Cancer recommends.

3.- Identity of the substance to be tested.

The identity, concentration, quality and pureness of the substance essayed is the client's responsability.

The day 5th of April, 1993 9 vials of 30ml of FR-91 substance were received at the Research and applied development Center S.A.L

The received substance was stored in the refrigerator. A contra sample of the essayed substance will be kept in the files of the Research and Applied Development S.A.L. during 10 years counted from date of the Final Report or until its caducity.

4.- Location of the study:

The study has been done at the stabulary and laboratories of the Pharmacology Department of the Research and Applied Development Center S.A.L. , Industrial Center Santiga, c/Argenters 6, 08130 Santa Perpetua de Mogoda(Barcelona)

5.- Dates of the study.

- 5.1 Signature of the protocol : September - 14th - 1992
- 5.2 Arrival of the substance
to be essayed : April - 5th - 1993
- 5.3 Arrivals of the animals : April -8th and 22nd-93
- 5.4 Start of the experimental
Work : April -28th - 1993
- 5.5 End of Experimental
Work : May - 31st - 1993
- 5.6 Report to the Client : see front page

6.- Material.

- 6.1 Animals
Male mice C57BL/6 and male mice B6D2F1 of about 20g
of weight coming from Charles River.
- 6.2 Reactives.
Cyclophosphamide (GENOXAL R, lot G4G4, Lab. FUNK)
Ethylic ether. Panreac.
Inoculation of tumoral cells of Lewis Lung carcinoma
given in by the Department of Càncer i Metastasi of
the Institut de Recerca Oncològica (IRO)(Barcelona).
Blue Trypan. Fluka.
Tripsina. Biological Industries.
PBS. Biomerieux.
- 6.3 Apparatuses
 - Bell of laminar flux TELSTAR type BIO-II-A
 - Scale, Mettler HA35
 - Inverted microscope KYOWA
 - Centrifuge JOUAN
 - Malassez Camera. Brand.
 - Autoclave Selecta.

7.- METHOD.

7.1 Lodging and identification of the animals.

The animals were lodged individually in cages Makrolon (29.5 x 12.4 x 12.0cm) with fixed floor and wood rasura .

The animals were identified individually through a perforation code in the ears.

At the label of each cage were the number of the Study, number of the treatment, dose, administration via, name of the Director of the Study, number and sex of the animal lodged and beginning of the treatment.

During all the essay the animals received standard A04C (Villemoisson sur Orge, France), lots 30107 and 30304.

The animals had water ad libitum.

The water is periodically analyzed to detect the presence of possible contaminants.

The temperature of the stabulary was of 22+- 2°C and the relative humidity of 50-70% reaching punctually the 75%

The illumination of the stabulary was distributed in 12 h of light and 12 h of darkness each 24 h.

7.2 Experimental Design.

After an adequate period of accomodation 11 male mice C57BL/6 were inoculated with tumoral cells of Lewis Lung procedent from de Department of Càncer i Metastasi of the Institut of Recerca Oncològica (IRO) (Barcelona)

The inoculation was done injecting through intramuscular via 0.5 ml to each animal that contained approximately 10 el. to 6 viable cells. The counting of the viable cells was done in camera of Malassez after a tinction with blue Trypan.

After 14 days the animals were sacrificed through ethylic ether inhalation . The primary tumor was taken out and the vegetative part was separated from the necrotic and muscular part.

The vegetative part corresponding to a pool of all the tumors was homogeneized. The cells were washed with PBS (phosphate tampon).

Following the latter a chemical disaggregation was done, treating the cells with trypsin at 0.25% (10 minutes, 37°C).

After a filtration through a sterile gase, it was centrifugated for 10 minutes at 1000 r.p.m. being decantated the the overswimming and resuspending in PBS.

After this, the counting of the viable cells/dead cells was done with the microscope and the Malassez camera, using the vital colorant blue Trypan.

All the process of extraction of the tumor and preparation of the inocul was done in laminar flux cabin and with sterile material.

Mice B6D2F1 were inoculated with the extracted tumoral cells to make the tumor spreading, following the same procedure.

After 12 days the definitive essayed started, inoculating 88 male mice B6D2F1. Being the technique the one described previously. Each animal was injected by intramuscular via in the right leg with 0.25 ml of suspension of cells that contained 10 viable cells.

The day of the inoculation is considered the day 0 of the essay.

Afterwards the animals were distributed in 5 experimental groups.

Group	Treatment	Dose i.p.		Colour
A	CONTROL	-	24	White
B	CICLOPHOSPHAMIDE	20 mg/kg	16	Yellow
C	FR-91	0.1 ml/animal	16	Blue
D	Fr-91	0.3 ml/animal	16	Green
E	Fr-91	0.5 ml/animal	16	Red

The animals received the corresponding treatment through intraperitoneal via from the day 1 to the day 11 of the study, including both.

The animals of the Control Group were administrated phisiologic serum.

As a reference substance ciclophosphamide was used (20 mg/kg) dissolved in phisiologic serum.

In groups A and B the volume of administration was of 10 ml/kg.

In order to obtain the ponderal evolution, the animals were weighed daily (day 0 to 21 of the study).

The day 21 of the study the animals were sacrificed through inhalation of ethilic ether. The primary tumor and the lungs

were extracted and weighed. The lungs were weighed in fresh and hydrated. The lobules were separated, the number and weight of the lung metastasis were counted. The weight of the metastasis was calculated considering the spheres 1,2,3,4 or 5 mm of diameter and applying the phormula $\frac{4}{3} r^3$ the weight in miligrams was obtained.

Micrometastasis were not counted.

8.- EVALUATION OF THE RESULTS.

The values obtained for each group were averaged in the following parameters: body weight, primary tumor weight, lungs weight and weight of the lung metastasis and the standard deviation was calculated and also the standard error of the average.

The results obtained in the different groups were compared through the varianza of a factor (3) and through the test of multiple comparisons of Duncan-Kramer (4) (5) (6).

In table n°1 the letters A,B,C,D and E represent the averages of each group and they are organized from minus to more.

Where this test appears, the differences between the two averages underlined by the same line is not statistically significant.

For the parameters "weight of the primary tumor" and "weight of lung metastasis" the percentage of inhibition obtained through each treatment in relation with the control group has been calculated following the phormula:

$$\text{inhibition \%} = \frac{\text{Wweight C} - \text{Weight T}}{\text{Weight C}} \times 100$$

where weight C is the average value of the weights obtained in the animals of the control group and weight T is the average value obtained in the animals of the treated group.

9.- FILES.

The experimental pages, results obtained and all the documents that refer to the Study will be kept at least during 10 years in an adequate file system, following the Normalized Procedures of Work of the Center of Research and Applied Development.

The material concerning this Study will not be distroyed without the written notification to the client.

10.- PROCEDURES OF WORK.

All the operations were done as it is described in the Normalized Procedures of Work of the Center of Research and Applied Development, S.A.L.

11.- DEVIATIONS TO THE PROTOCOL.

In order the number of tumoral cells inoculated be 10 viable cells, the volume of administration was increased from 0.15 ml to 0.5 ml in the phase of propagation of the tumor and from 0.2 ml to 0.25 in the phase of inoculation of the tumor, to the animals in which the treatments were essayed.

12.- RESULTS.

The global results are found in Table nº1 and the individual data, in tables nº 3,4,5,6 and 7.

12.1 Observations.

The day 12 of the study the animal nº 52 belonging to the group treated with FR_91 at a dose of 0.1 ml/animal appeared dead and the day 5 of the study the animal 68, treated with Fr-91 at a dose of 0.3 ml/animal, for which in the elaboration of the results no value belonging to the mentioned animals has been taken into account.

The cause of death could not be determined because the tissues were autolytic.

The day 20 of the study, the animal 80, belonging to the group treated with FR-91 at a dose of 0.5 ml/animal died. With this animal the same procedure followed with the other animals sacrificed the day 21 of the study was followed, so its values are included in the results.

12.2 Ponderal evolution.

In table nº2 the ponderal evolution of the animals throughout the study can be observed.

Starting the day of the inoculation of the tumor (day 0) the animals of all groups suffered a decrease of weight, that they were recovering in the measure time passed.

Since the aparition of the tumor the weight of the animals increases faster than in all the groups.

12.3 Weight of the tumor.

Starting from the day 6 of the Study the aparition of the tumor can be observed.

In all animals the tumor was correctly set.

In the animals of the Control group and of the groups treated with FR-91 at the doses of 0.1, 0.3, and 0.5 ml per animal i.p., the weight of the primary tumor was similar, without finding significant statistical differences among the mentioned groups.

The administration of CICLOPHOSPHAMIDE at doses of 20 mg/kg i.p. produced an inhibition of the weight of the primary tumor of a 15.4% in relation with the Control group.

Significant statistical differences were observed between the weight of the primary tumor treated with CICLOPHOSPHAMIDE and the rest of the groups.

12.4 Weight of the lungs.

As it can be observed in table n°1, there is a correlation between the weight of the lungs of each group and the number of metastasis observed.

The groups treated with the substance of the reference, CICLOPHOSPHAMIDE, and the substance FR-91 at a dose of 0.5 ml/animal have shown significant statistical differences in relation with the Control group.

12.4 Number and weight of lung metastasis.

The number of lung metastasis of each animal can be observed in tables n°3, 4, 5, 6, and 7.

The evaluation of the lung metastasis has been done transforming the number and size of the lung metastasis into weight in miligrams.

The weight of the lung metastasis of each group can be observed in table n°1.

The administration of the substance of the reference, CICLOPHOSPHAMIDE, produced an inhibition of the lung metastasis of 97.6% in relation with the Control group.

Significant statistical differences were found between the group treated with CICLOPHOSPHAMIDE and the rest of the groups.

The administration of the substance FR-9 at the doses of 0.1, 0.3 and 0.5 ml per animal produced inhibitions of the weight of the metastasis of 8.2, 24.0 and 35.9% respectively, in relation with the Control group.

No significant statistical differences were found between those groups and the Control group.

CONCLUSIONS.

13.- The antitumoral activity of the substance Fr-91 has been evaluated at three levels of doses intraperitoneal via in the experimental model of Lewis Lung Carcinoma in the mouse.

In our experimental conditions, the antitumoral activity of the substance FR-91 has not been observed, although a decrease of the lung metastasis has been appreciated.