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ORIGINAL ARTICLE

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Insulin-induced enhancement of antitumoral response to methotrexate in breast cancer patients

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Abstract *Purpose*: It has been reported that insulin increases the cytotoxic effect in vitro of methotrexate by as much as 10,000-fold. The purpose of this study was to explore the clinical value of insulin as a potentiator of methotrexate. *Patients and methods*: Included in this prospective, randomized clinical trial were 30 women with metastatic breast cancer resistant to fluorouracil + Adriamycin + cyclophosphamide and also resistant to hormone therapy with measurable lesions. Three groups each of ten patients received two 21-day courses of the following treatments: insulin + methotrexate, methotrexate, and insulin, respectively. In each patient, the size of the target tumor was measured before and after treatment according to the Response Evaluation Criteria In Solid Tumors. The changes in the size of the target tumor in the three groups were compared statistically. *Results*: Under the trial conditions, the methotrexate-treated group and the insulin-treated group responded most frequently with progressive disease. The group treated with insulin + methotrexate responded most frequently with stable disease. The median increase in tumor size was significantly lower with insulin + methotrexate than with each drug used separately. *Discussion*: Our results confirmed in vivo the results of previous in vitro studies showing clinical evidence that insulin potentiates methotrexate under conditions where insulin alone does not promote an increase in tumor growth. Therefore, the chemotherapy antitumoral activity must have been enhanced by the biochemical events elicited in tumor cells by insulin. *Conclusions*: In multidrug-resistant metastatic breast cancer, methotrexate + insulin produced a significant antitumoral response that was not seen with either methotrexate or insulin used separately.

Keywords Breast Cancer · Chemotherapy · Insulin · Methotrexate · Tumor growth

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Introduction

It is known that slowly growing cancers have tumor cell populations with a low-growth fraction and are less sensitive to chemotherapy than rapidly growing tumors with high-growth fractions [11]. Slowly growing malignancies have relatively more cells in a noncycling status and fewer cells in a cycling status than rapidly growing malignancies. It has been demonstrated that insulin as a pharmacological agent induces the switch from a noncycling to a cycling status in tumor cells [5]. In MCF-7 human breast cancer cells, insulin has been shown to increase the cytotoxic effect of methotrexate up to 10,000-fold in vitro [1]. Ellipticine uptake is also increased by insulin [9]. It has been suggested that insulin is effective in potentiating most chemotherapy drugs. This insulin-induced potentiation has been

proposed as a strategy for breast cancer treatment, but confirmatory clinical trials are still lacking [2]. This study was carried out to confirm insulin-induced clinical potentiation of the antitumoral effect of methotrexate as suggested by preclinical studies and to establish a mechanism of action for this antitumoral effect.

Patients and methods

Patients

The study was conducted in 30 patients with breast cancer admitted to medical centers that reported medical data to the Cooperative Trials Center (CTC) of PharmaBlood, R&D Department, Florida. A prospective, randomized trial was carried out. All patients met the following eligibility criteria: histologically confirmed breast carcinoma, metastatic stage (M1); Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 2; age ≤ 74 years; and adequate hematological function (WBC count $\geq 4000/\mu l$, neutrophil count $\geq 2000/\mu l$, hemoglobin level ≥ 9.0 g/dl, platelet count $\geq 10 \times 10^4/\mu l$), renal function (serum creatinine ≤ 1.5 mg/dl, 24-h creatinine clearance ≤ 60 ml/min), liver function (total bilirubin ≤ 2.0 mg/dl, serum transaminases not more than twice the upper limit of the normal range), and respiratory function (PaO_2 ≥ 60 Torr). The patients included had measurable lesions, as required by the Response Evaluation Criteria In Solid Tumors (RECIST) system of tumor assessment [13], and if they had a positive estrogen receptor status, they had been treated with and become resistant to hormone therapy.

All patients included in the study had progressive disease (RECIST criteria) after chemotherapy with at least four series of fluorouracil + Adriamycin + cyclophosphamide (FAC) and had not been treated with any other chemotherapy. They were randomly allocated to three groups of ten patients each: group 1 was treated with insulin + methotrexate as described below, group 2 was treated with methotrexate without insulin, and group 3 was treated with insulin without methotrexate. Written informed consent, including detailed information about risks and benefits, was approved and signed by all the patients included in the study. Central computerized remote randomization was performed, with patients being allocated to one of the groups through random sequence generation by the permuted block method. An assessment of the results after 30 patients had completed the trial showed that this sample size was enough. The patients were recruited from two oncological medical centers in Montevideo, Uruguay (first at the National Cancer Institute and then at Interdoctors Medical Center), both of which participated with their data in the network operated and sponsored by the Cooperative Trials Center (CTC) of PharmaBlood R&D Department.

The institutional ethics committee of PharmaBlood and the institutional review boards of the participating medical centers approved the trial. The ethical reviewers considered that an 8-week delay before starting second-line chemotherapy after FAC had failed in all the patients included in the trial was acceptable. This determination was consistent with the standard of care in this clinical situation which has been recently well summarized [3]:

> Despite almost 30 years of clinical cancer research, the true impact of second and subsequent lines of chemotherapy on the outcome of metastatic breast cancer patients, especially on the duration of survival, is still unknown. In the virtually incurable metastatic setting, issues like quality of life and patients' preferences gain particular relevance.

The accepted protocol was resubmitted to the committee for review in order to obtain approval for treatment of patients with insulin alone considering the potentially harmful effect through the activation of receptors for insulin/insulin-like growth factors. The committee confirmed the approval on the basis of reports of no harmful effect of this treatment [6, 7]. The results of the study confirmed the committee's criteria because no significant differences were found in tumor growth either between the insulin-alone group and the methotrexate-alone group or between before and after treatment in the insulin-alone group.

Treatment

All the patients included in the study received two 21-day courses of treatment separated by a 7-day interval without treatment between courses. In group 1, the treatment course was intravenous human recombinant insulin (0.3 U/kg body weight every other day) followed 20 min later by a 15-min intravenous infusion of methotrexate (2.5 mg/m^2 in 50 ml 30% glucose). If symptomatic hypoglycemia was observed, the 30% glucose solution containing methotrexate was infused immediately. An oral glucose supplement was also prescribed to prevent delayed hypoglycemic symptoms. In group 2, insulin was omitted and methotrexate was administered intravenously at the same dose and in the same solution (2.5 mg/m^2 in 50 ml 30% glucose) as in group 1. In group 3, methotrexate was omitted, insulin was administered at the same dose as in group 1, and 30% glucose solution was also administered intravenously 20 min after insulin or sooner if hypoglycemic symptoms were evident.

Tumor growth assessment

After 8 weeks (two 3-week courses plus 1 week interval after each course), the response to treatment was assessed in each patient using RECIST criteria [13]. The sum of the longest diameter of measurable target lesions and the number of non-target lesions were recorded immediately before and after this 8-week period. Skin nodules and palpable lymph nodes were measured using calipers. Lung and liver target lesions were measured by a CAT scan. Responses were confirmed by repeating the assessment 4 weeks after status assignment. Three independent reviewers performed all image measures (Telemedical Organization, North Miami Beach, Fl.).

The distribution of RECIST status (progressive disease, stable disease, or remission) in each group was recorded. This distribution was dependent on treatments that showed statistical significance according to the Chi-squared test. The data from the RECIST measurements of the change in tumor size of the patients in each treatment group, expressed as a percentage of pretreatment measurements, were compared using Student's *t*-test. Additionally, increases in tumor size were expressed as a proportion of the initial value and analyzed by the two-proportion test comparing pairs of groups: group 3 vs group 1, and group 2 vs group 1. The sample size was assessed after analysis of the results when the trial was finished for the 30 patients allocated to the three groups. The above pairs of groups were analyzed for the proportion of progressive disease in each. Ten patients in each group was the required sample size for an 80% chance of rejecting the hypothesis of equal proportions at the 0.05 level of significance when the true proportions were those shown by the study. Statistical analysis was performed using StatsDirect software and an independent expert was consulted.

Results

The characteristics of the patients included are shown in Table 1. The three groups were comparable in the most relevant prognostic parameters for the clinical condition studied. Previous treatments were also comparable. The similar range of sizes of target lesions measured before treatment was especially significant, allowing the change in size to be measured as a percentage of initial size.

Table 1 Clinical characteristics of the 30 women with metastatic breast cancer (M1) included in the three treatment groups

	Group 1 (insulin + methotrexate)	Group 2 (methotrexate)	Group 3 (insulin)
No. of patients	10	10	10
Age range (years)	42–64	44–68	39–69
< 50 years	4	3	4
Estrogen receptor-positive	7	7	6
Progesterone receptor-positive	7	5	7
Measurable M1			
Lung	6	4	4
Liver	1	2	2
Skin	2	2	3
Lymph nodes	1	2	1
Range of initial (pretreatment) RECIST sum of target measures (mm)	57–65	59–64	56–66

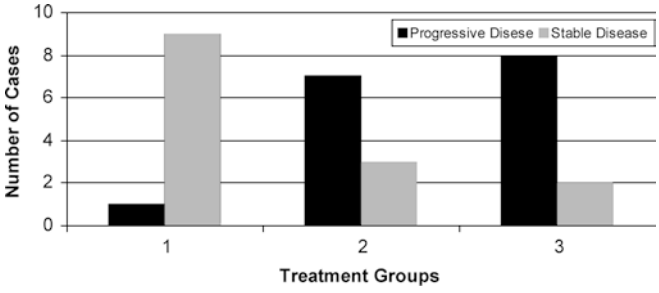

Fig. 1 Post-treatment RECIST status of measurable target lesions. After the respective treatment, the change in the measurable lesions selected as targets in each patient was evaluated and the status of therapeutic response, defined in terms of the RECIST criteria, was recorded. Under the conditions of this study, two response statuses were recorded: stable disease (less than 20% increase or less than 30% decrease in the sum of largest diameters of targets) and progressive disease (more than 20% increase in the sum of diameters). Stable disease, the best response obtained, was more frequent in the group treated with insulin + methotrexate (nine of ten) than in methotrexate-treated group (three of ten) or insulin-treated group (two of ten). The distribution of RECIST type responses (stable disease or progressive disease) was dependent on the treatments tested and statistically significant ($P < 0.01$, Chi-squared test)

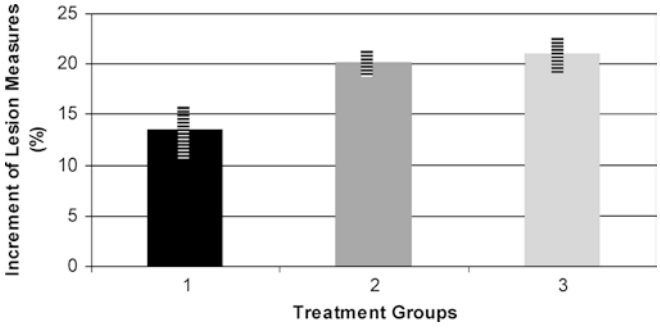

Fig. 2 Increase in size of measurable target lesions (RECIST assessment). After each treatment, the change in the measurable lesions selected as targets in each patient was evaluated in terms of the RECIST criteria and expressed as a percentage of the measured pretreatment size. For each treatment group, the mean ± SD and 95% CI for the values of this response were calculated: group 1 (insulin + methotrexate) 13.51 ± 3.01% (95% CI 11.35–15.67%); group 2 (methotrexate) 20.21 ± 2.27% (95% CI 18.58–21.84%); group 3 (insulin) 21.04 ± 2.17% (95% CI 19.49–22.59%). The increase in size of lesions in group 1 (insulin + methotrexate) was significantly lower (Student's *t*-test) than the increase in size in group 2 (methotrexate) ($P < 0.001$) and group 3 (insulin) ($P < 0.001$). Group 2 showed no significant difference from group 3 ($P = 0.41$)

Figure 1 shows the RECIST status assessed under the study conditions. Progressive disease was the most frequent response in two of the three groups: in group 2 (treated with methotrexate alone) there were seven progressive disease and three stable disease, and in group 3 (treated with insulin alone) there were eight progressive disease and two stable disease. In group 1 (treated with insulin + methotrexate), stable disease was the most frequent response (nine stable disease, one progressive disease). The distribution of RECIST type responses (stable disease and progressive disease) was dependent on the treatments tested, and was statistically significant ($P < 0.01$, Chi-squared test).

Figure 2 shows the means and 95% confidence intervals (CI) of the percentage increase in tumor size after treatment in the three groups. Increases in tumor size were significantly lower in patients treated with insulin + methotrexate than in those treated with insulin alone and significantly lower than in those treated with methotrexate alone.

From the same set of measurements, Figs. 1 and 2 show the clinical and biological effects of the treatments, respectively. Figure 1 indicates that the decrease in tumor growth induced by insulin + methotrexate reached the level of a clinically confirmed antitumoral response because more patients in this group achieved stable disease. Figure 2 shows that insulin + methotrexate treatment reduced tumor growth. All patients completed the study. Hypoglycemia was induced in all patients receiving insulin as part of their protocol. Eight patients in group 1 and nine patients in group 3 showed no hypoglycemic symptoms during the 20 min after insulin injection; they showed a mean blood glucose level of 456 mg/dl (range 376–520 mg/dl). Two patients in group

Table 2 Maximum recorded WHO toxicity grade in the patients included in the trial comparing insulin + methotrexate (group 1), methotrexate (group 2) and insulin (group 3). The numbers of patients with each toxicity grade (0 to 4) in the three groups are shown. No other toxicities referred to in the WHO criteria were recorded

Toxicity	Grade				
	0	1	2	3	4
Erythrocytes					
Group 1	10	0	0	0	0
Group 2	8	2	0	0	0
Group 3	10	0	0	0	0
Leukocytes					
Group 1	10	0	0	0	0
Group 2	6	3	1	0	0
Group 3	10	0	0	0	0
Platelets					
Group 1	10	0	0	0	0
Group 2	9	1	0	0	0
Group 3	10	0	0	0	0
Mucositis					
Group 1	8	2	0	0	0
Group 2	4	3	3	0	0
Group 3	10	0	0	0	0

1 and one patient in group 3 showed hypoglycemic symptoms within 20 min of insulin injection (13, 16 and 19 min), but recovered immediately after starting the glucose infusion. There was no evidence of any harmful sequelae attributable to the hypoglycemia induced.

Table 2 shows the toxicities associated with antitumoral chemotherapy (according to WHO criteria) recorded in this study.

Discussion

The methotrexate dose used in this study was chosen because a similar dose of methotrexate had been used previously in patients receiving low-dose combined chemotherapy potentiated with insulin [2]. In addition, the cumulative monthly dose was no higher than the monthly dose used in the well-known standard protocol of methotrexate + fluorouracil + cyclophosphamide (CMF). Indeed, each individual methotrexate injection (2.5 mg/m^2) was less than the dose usually considered optimal in non-potentiated protocols but is within the presumed range of effective dose for a potentiation similar to the one observed in vitro. The results of this study confirmed the expected safety of the selected methotrexate dose. The toxicities in the methotrexate-alone group were not relevant (WHO grades 1/2) and they were even lower when methotrexate was associated with insulin, only producing a grade 1 mucositis. In this study, methotrexate at this safe low dose did not have an antitumoral effect when used alone (group 2), but it did produce a significant antitumoral effect when administered after insulin (group 1). The term antitumoral is used here as a description of the clinical effect of a reduction in the proportion of patients showing progressive disease.

Therefore, as reported previously, our results support the hypothesis that insulin can potentiate the antitumoral effect of methotrexate [2] and confirm in vivo previously reported in vitro results [10]. Our results also show insulin potentiation of methotrexate in this condition, where insulin alone did not promote an increase in tumor growth (group 3). This effect is in agreement with previous results from in vitro models where insulin enhancement of cytotoxicity was not a direct consequence of an insulin-dependent increase in the growth rate of tumor cells [1, 10]. The same in vitro models do not allow an explanation of the insulin potentiation of methotrexate in terms of the known effects of insulin treatment upon the specific metabolism of methotrexate which include a decrease in intracellular pH induced by glucose metabolism and tight binding of the drug to its target, dihydrofolate reductase. Insulin potentiation of other antitumoral drugs has been reported [9].

If we discount the promotion of tumor cell growth and the interaction with the specific target as the mechanism of potentiation of methotrexate by insulin, we can hypothesize that this mechanism could involve another general insulin-dependent biochemical pathway as has been previously suggested to explain the in vitro potentiation of methotrexate by insulin [1]: protein synthesis in tumor cells is one of the biochemical pathways activated by insulin [8]. Most chemotherapy drugs that have been tested using insulin to increase cytotoxicity are known modifiers of protein structure that act at the genetic or epigenetic level [12]. High levels of mutated or epigenetically modified proteins could be responsible for the cytotoxic mechanism elicited by the insulin-dependent increase in protein synthesis associated with chemotherapy drugs. The relative selectivity of this mechanism of action for insulin + methotrexate in malignant cells is attributed to the agonism of insulin and insulin-like receptors in tumor cells. Certainly, the response to insulin is more intense in most tested cancer cells than in most normal cells. This is probably because cancer cells are richer in receptors for insulin-like growth factors that are cross-stimulated by insulin [4].

Conclusion

The in vitro potentiation of methotrexate cytotoxicity by insulin in human breast cancer cell lines was previously known. We report the results of a randomized, controlled trial that confirmed, at the clinical level, the potentiation by insulin of the antitumoral effect of methotrexate in women with advanced breast cancer. The term antitumoral is used as a description of the clinical effect of a reduction in the proportion of patients with progressive disease. Under the conditions of this study, the dose of insulin used did not increase tumor growth. Therefore, we suggest that, as has been reported

in vitro, methotrexate potentiation by insulin was not a direct consequence of the expansion of the tumor cycling cell population but a consequence of some of the biochemical events that are simultaneously activated. The enhancement of methotrexate uptake by tumor cells and/or the promotion of protein synthesis in a mutagenic intracellular environment are hypothesized to be mechanisms of potentiation. It is known that both events are promoted by insulin acting as a cross-agonist of the highly expressed receptors for insulin-like growth factors in breast cancer cells.

These mechanisms, which are shared with other primary tumor cells and with other chemotherapeutic agents suggest that it would be worthwhile to pursue further study of these phenomena in other tumors and with other chemotherapeutic agents.

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