ANTICANCER RESEARCH *25*: 2229-2234 (2005)

# Antileukemic Activity of Glycated Fibrinogen-Methotrexate Conjugates

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**Abstract.** *Background: The aim of the study was to compare the antileukemic activity of methotrexate (MTX) conjugates with native and glycated fibrinogen. We expected that conjugates based on glycated fibrinogen would reveal higher antileukemic activity because of decreased plasmin digestibility and a higher retention rate of glycated fibrinogen in the body. Materials and Methods: Fibrinogen was glycated using a high-temperature procedure at 65-85˚C. Glycated fibrinogens were examined with respect to their ability to clot and susceptibility to plasmin digestion. Native fibrinogen (F) and fibrinogens glycated at 65 and 73˚C (F65 and F73) were conjugated with MTX and tested in mice bearing P388 leukemia, at a dose of 40 mg of MTX per kg of body weight. Results: Glycated fibrinogens retained their ability to clot. Compared to native fibrinogen, they were more resistant to digestion by plasmin. All tested conjugates revealed higher antitumor activity than the free drug. Increases in average lifespan over the control group were 34% for free MTX, 137% for F-MTX, 151% for F65-MTX and 91% for F73-MTX. The differences between the antitumor activities of all conjugates were not statistically significant. Conclusion: It seems necessary to compare the antitumor activities of MTX conjugates based on native and glycated fibrinogen in different tumor models, to demonstrate the expected differences.*

Fibrinogen is one of the main components of tumor stroma. The deposits of fibrinogen result from the characteristic hyperpermeability of tumor microvasculature, which contributes to the local extravasation of plasma fibrinogen. Much of the extravasated fibrinogen is rapidly clotted to fibrin by procoagulants associated with tumor cells or originating from other tissues (1-3). The phenomenon of local fibrin deposition and dissolution suggests that fibrinogen might be used as a carrier of anticancer drugs. Our previous experiments confirmed that native fibrinogen is a promising antitumor drug carrier. Fibrinogen-methotrexate (MTX) conjugates exhibited a lower *in vitro* cytotoxicity compared to MTX alone, as well as a significantly higher *in vivo* antitumor activity in mice bearing P388 leukemia (4). Hirakawa *et al.* applied fibrin glue containing MTX in the local therapy of brain tumors. The *in vivo* study showed that MTX-fibrin therapy of rats bearing 9L-gliosarcoma resulted in the inhibition and, in some cases, in the disappearance of the tumor. In the clinical study, sustained release of MTX lasting more than one week was found, and tumor shrinkage was noted in patients with brain tumors (5).

Glycation is a non-enzymatic reaction between reducing sugars and proteins. In the first stage, the carbonyl group of the carbohydrate reacts with the amino group of the protein. The resulting labile Schiff base undergoes Amadori rearrangement to form a stable Amadori product (6). Glycation is a process which occurs under physiological and pathological conditions in humans. It contributes to the modifications of proteins and their properties. Glycated proteins are characterized, among others, by decreased digestibility (7). Fibrin prepared from glycated fibrinogen exhibits a significant resistance to plasmin degradation (8, 9).

We were interested in how glycation would influence the therapeutic utility of fibrinogen-MTX conjugates. We expected that glycated fibrin containing a chemically-bound antitumor drug would be more resistant to plasmin digestion and that the drug would be present in the

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*Key Words:* Fibrinogen, methotrexate, conjugates, glycation, antitumor activity.

proximity of the tumor for a longer time. This hypothesis is supported by the results of Murakami *et al.*, who showed that the retention rate of glycated fibrinogen in some tissues (renal cortex) is higher than that of the unglycated form (9).

In previous reports, we presented a method, called high-temperature glycation, which makes use of the glycation reaction and enables easy attachment of reducing sugars to proteins without any prior modification or chemical activation (10, 11). The carbohydrate is mixed with the protein, the mixture is lyophilized and briefly heated (up to 120˚C). Running the reaction in semi-water-free conditions allows a significant reduction in the coupling time. The conjugates obtained by this procedure are stable over a wide range of pH and in the presence of dissociating substances. Depending on the temperature, the method provides different levels of substitution (10, 11). In the case of glycation of bovine serum albumin by D-glucose, the average level of substitution obtained at 50˚C was 4.7 mol glucose/mol BSA and reached 53 moles of glucose per mol BSA at 104˚C (12). The reaction conditions do not affect the biological activity of the proteins. Lyophilized antibodies and enzymes can be heated to very high temperatures without loss of activity (11). Using lysozyme as a model protein, it was shown by ESI-MS and circular dichroism that the high temperature of glycation does not influence protein conformation (13). Immunochemical analyses showed that larger proteins, such as fibrinogen, also retained their conformation during heating in water-free conditions (14).

In the present report, high-temperature glycation was used to obtain glycated fibrinogen. We examined how that reaction affected the biological functions of fibrinogen, its ability to clot and susceptibility to degradation by plasmin. Further, we decided to examine the antitumor activity of glycated fibrinogen-MTX conjugates and to compare it with the activity of free drug and unglycated fibrinogen-MTX conjugate in an *in vivo* mouse leukemia model.

## Materials and Methods

D-glucose was obtained from POCh (Gliwice, Poland). Bovine fibrinogen and bovine thrombin were purchased from Biomed (Lublin, Poland) and human plasmin from Kordia (Leiden, Netherlands). N-hydroxysuccinimide (NHS) and N,N'-dicyclohexylcarbodiimide (DCC) were obtained from Fluka (Buchs, Switzerland) and MTX from Lachema (Brno, Czech Republic).

*Glycation of fibrinogen.* The fibrinogen was dialyzed against 0.001 M $Na_2HPO_4$, pH 8.3. The dialyzed protein was mixed with D-glucose dissolved in the same buffer. The mixture was divided into 1-ml aliquots. Each sample contained 1.5 mg of fibrinogen and 4.5 mg of D-glucose. The samples were frozen at –60˚C and lyophilized. The lyophilizates were heated at various temperatures, up to 85˚C, for 30 min and then dissolved in 750 μl of aqueous solutions.

*Clotting assay.* The clotting assay was carried out according to the method previously described by Tuan *et al.* (15), with some modifications. Each sample of fibrinogen or conjugate was diluted 5 times with 0.1 M Tris-HCl, 3.0 mM $Ca(NO_3)_2$, pH 8.3. To 1.2 ml of 0.91 μM fibrinogen or conjugate, 50 μl of thrombin (7 U/ml 0.1 M Tris-HCl, 3.0 mM $Ca(NO_3)_2$, pH 8.3) was added. The reference samples contained 50 μl of buffer instead of thrombin. After incubation at 37˚C for 30 min, the samples were centrifuged at 14,000 x g and the concentrations of nonclottable protein in the supernatants were measured by spectroscopy at 280 nm. The amount of clottable protein was calculated by subtracting the amount of protein in the supernatant of the sample with thrombin from the amount of protein in the supernatant of the control sample, which contained the same amount of conjugate or fibrinogen, but did not contain the thrombin. Because glycated fibrinogen is more soluble in alkaline conditions, an increased pH (8.3 instead of 7.5) of the clotting reaction was used to avoid spontaneous formation of microaggregates.

*SDS-PAGE.* SDS-polyacrylamide gel electrophoresis (PAGE) of glycated fibrinogens was carried out in 8% gel under reducing conditions, according to Laemmli (16). SDS-PAGE of the plasmin degradation products of fibrinogen and conjugates of fibrinogen were carried out in 8% gel under non-reducing conditions, also according to Laemmli.

*Proteolytic digestion of glycated fibrinogens by plasmin.* 0.05 mg of plasmin in 10 μl of 0.05 M HEPES-HCl, 0.05 M sodium acetate, pH 8.5, was added to 200 μl of α 0.46 μM solution of fibrinogen or conjugate in 0.02 M Tris-HCl, 5 mM $CaCl_2$, pH 7.4. The samples were incubated at 37˚C. After 1, 2, 3.5 and 24 h, 10 μl was taken from each sample. The reaction was quenched in these aliquots by the addition of 5 μl 0.3 M ε-aminocaproic acid. The aliquots were mixed with loading buffer without 2-mercaptoethanol and analyzed by SDS-electrophoresis in non-reducing conditions.

*Conjugation of MTX with fibrinogen and glycated fibrinogen.* In the first step, the MTX anhydride was prepared in the reaction of the acid form of MTX (4) and DCC, at the molar ratio 1:0.9. The reaction was carried out in dimethylformamide (DMF) at 4˚C for 48 h in darkness. The mixture was then centrifuged to remove dicyclohexylurea and lyophilized. The lyophilizate was washed several times with dichloromethane to remove the unreacted DCC. The resulting pure MTX anhydride was dissolved in DMF and mixed with NHS (the molar ratio 1:3). The esterification reaction was carried out for 2 h at 4˚C in darkness. The resulting monoactive ester was added drop-wise under the surface of the stirred fibrinogen solution in 0.1 M $NaHCO_3$. The reaction was allowed to proceed for 2 h in darkness at 4˚C. Free MTX was removed by extensive dialysis for 48 h against 0.1 M $NaHCO_3$.

*Mice.* Twelve- to 16-week-old DBA/2 and (C57Bl/6 x DBA/2)F1 male mice, weighing 20-25 g, were used. All mice were supplied by the Animal Breeding Center of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland and maintained in standard laboratory conditions. All experiments were performed according to the *Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education* issued by the New York Academy of Sciences' *Ad Hoc* Committee on Animal Research and were approved by the First Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

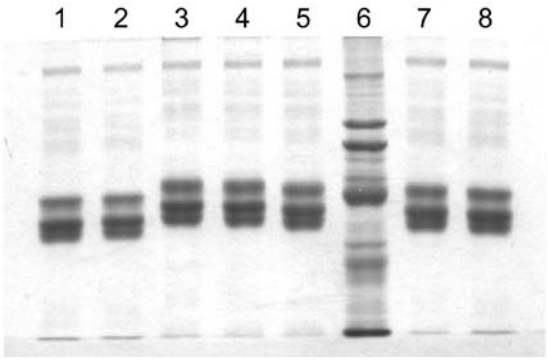


Figure 1. *Effect of temperature on the glycation of fibrinogen. SDS-PAGE in 8% gel in the presence of 1% 2-mercaptoethanol (16). Lane 1: native bovine fibrinogen; lane 2: fibrinogen co-lyophilized with D-glucose, not heated; lane 3: fibrinogen-D-glucose conjugate, reaction at 85˚C; lane 4: fibrinogen-D-glucose conjugate, reaction at 80˚C; lane 5: fibrinogen-D-glucose conjugate, reaction at 75˚C; lane 6: standard of molecular weights (205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa); lane 7: fibrinogen-D-glucose conjugate, reaction at 70˚C; lane 8: fibrinogen-D-glucose conjugate, reaction at 65˚C.*

*Antitumor activity assay in vivo.* The passages of leukemia in DBA/2 mice, as well as the experiments in $F_1$ hybrids, were carried out according to the NIH/NCI standard screening protocols *in vivo* (17, 18). The mice were inoculated with $10^6$ leukemia (P388) cells *i.p.* and, 24 h later, each mouse was injected with the appropriate agent (*i.p.*). The treatment dose was 40 mg of MTX per kg of body weight. Three different MTX conjugates were examined: i) with native bovine fibrinogen (F-MTX), ii) with fibrinogen glycated at 65˚C (F65-MTX), and iii) with fibrinogen glycated at 73˚C (F73-MTX). The average level of substitution of all conjugates was 11.4 moles of MTX per mole of the protein. Animals were randomly divided into 5 groups: 1) untreated control, and 2-5) mice treated respectively with MTX, F-MTX, F65-MTX and F73-MTX. The antitumor effect was evaluated using the following parameters: a) Increase in lifespan of treated mice over the control (ILS%), calculated from the following formula: $(AST_T/AST_C) \times 100 - 100\%$, where $AST_T$ is the average survival time of treated animals, and $AST_C$ is the average survival time of untreated control mice. b) Long-term survivors (LTS) – number (and/or precentage) of mice which survived tumor-free for at least 2 months.

*Statistical evaluation.* The Cox-Mantel test was used for evaluation of the data from *in vivo* experiments. A $p$ value $<0.05$ was considered significant.

## Results

*Glycation of fibrinogen.* The high-temperature glycation method (11) was used to conjugate D-glucose with bovine fibrinogen. Reactions were conducted in water-free conditions for 30 min at temperatures up to 85˚C. The obtained samples were analyzed by SDS-PAGE (Figure 1). The reaction temperature had a substantial effect on the efficiency of glycation. The average level of substitution obtained at 65˚C was 58 mol glucose/mol fibrinogen and gradually increased with temperature. At 85˚C, a level of substitution as high as 164 mol glucose per mol fibrinogen was achieved. The levels of substitution estimated using SDS-PAGE are listed in Table I. At temperatures higher than 85˚C, the insoluble yellow products were formed and a color change of the sample was observed. Fibrinogen lyophilized with glucose, without thermal processing, was indistinguishable in SDS-PAGE from the control bands of native fibrinogen. Lyophilized fibrinogen heated without glucose was also indistinguishable from the control bands of native protein (data not shown).

Table I. *Properties of glycated fibrinogen.*

| | Reaction temperature (˚C) | Mol "glucose"/mol fibrinogen | Clottability (%)±SD |
|---|---|---|---|
| lyophilized fibrinogen | - | - | 77.1±1.9 |
| fibrinogen lyophilized with glucose, not heated | - | - | 81.0±2.1 |
| fibrinogen heated at 85˚C | - | - | 75.7±0.2 |
| fibrinogen-glucose conjugate | 65 | 58 | 89.2±0.5 |
| fibrinogen-glucose conjugate | 70 | 93 | 87.2±2.0 |
| fibrinogen-glucose conjugate | 75 | 97 | 86.8±3.6 |
| fibrinogen-glucose conjugate | 80 | 130 | 73.3±7.7 |
| fibrinogen-glucose conjugate | 85 | 164 | 57.7±9.8 |

*Clottability of glycated fibrinogens.* The glycation reaction affected, to some extent, the ability of fibrinogen to clot (Table I). The clottability of glycated bovine fibrinogen gradually decreased with increasing glycation temperature. However, in the case of fibrinogens glycated at 65, 70 and 75˚C, the clottability was higher than that of the native fibrinogen. The ability of fibrinogen glycated at 80˚C to clot was similar to that of the native protein, while the ability of fibrinogen glycated at 85˚C to clot was much lower. The thermal processing of fibrinogen lyophilized without glucose did not significantly influence its clotting activity. Native fibrinogen was found to contain 77.1±1.9% of clottable protein, whereas the value for heated fibrinogen was 75.7±0.2%. The clottability of fibrinogen lyophilized with glucose was higher than that of native fibrinogen lyophilized without glucose.

The clotting abilities (clottabilities) of native and glycated fibrinogens conjugated with MTX were also tested. The F-MTX conjugate contained 53.6% of clottable fibrinogen. The clottability of the F65-MTX conjugate was 39.7%, while F73-MTX almost completely lost the ability to clot (3.2% clottability).

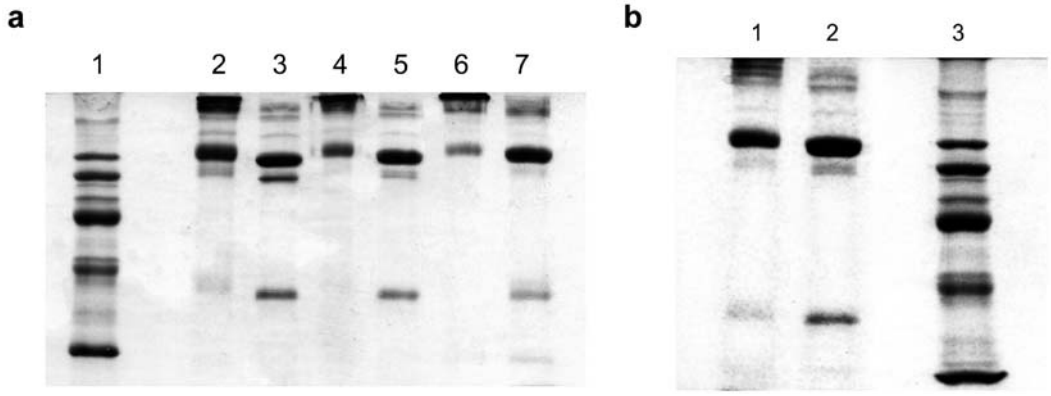


Figure 2. *Proteolytic degradation of native fibrinogen and glycated fibrinogen by plasmin. SDS-PAGE in 8% gel, non-reducing conditions. (a) Lane 1: standard of molecular weights (205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa); lane 2: fibrinogen glycated at 72˚C digested for 24 h; lane 3: native fibrinogen digested for 24 h; lane 4: fibrinogen glycated at 72˚C digested for 3.3 h; lane 5: native fibrinogen digested for 3.3 h; lane 6: fibrinogen glycated at 72˚C digested for 0.5 h; lane 7: native fibrinogen digested for 0.5 h. (b) Lane 1: fibrinogen glycated at 62˚C digested for 2 h; lane 2: native fibrinogen digested for 2 h; lane 3: standard of molecular weights (205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa).*

Table II. *Mean survival time of tumor (P388)-bearing mice treated with free MTX or with different conjugates.*

| Group | N | ILS % | Mean ± SD | S | L |
|---|---|---|---|---|---|
| Control | 6 | | 11.2±1.3 | - | - |
| MTX | 6 | 34 | 15.0±0.6 | - | - |
| F-MTX conjugate | 6 | 137 | 26.5±14.8 | - | 1 |
| F65-MTX conjugate | 6 | 151 | 28.0±6.0 | 1 | - |
| F73-MTX conjugate | 6 | 91 | 21.3±10.5 | - | 1 |

N - number of mice
ILS - increase in lifespan
Mean ± SD - mean survival time (days) ± standard deviation
S - long-term surviviors – number of mice which survived 2 months or more after tumor cells inoculation without clinical signs of leukemia
L - number of mice which died before control (toxicity).

*Proteolytic degradation of fibrinogen and its conjugates by plasmin.* The SDS electrophoresis patterns of native fibrinogen and fibrinogen glycated at 72˚C and digested for 0.5, 3.3 and 24 h are shown in Figure 2a, while these of fibrinogen glycated at 62˚C and of native protein digested for 2 h are presented in Figure 2b. Glycated fibrinogens are more resistant to digestion by plasmin than native protein. The difference between the extent of fibrinogenolysis of native fibrinogen and fibrinogen glycated at 62˚C is less evident than that between native fibrinogen and fibrinogen glycated at higher temperature (72˚C).

*Antitumor activity.* The antitumor activity of MTX conjugates was tested with three forms of fibrinogen: glycated at 65˚C, at 73˚C and native. In our experiment, all conjugates revealed higher antitumor activity *in vivo* (expressed as ILS) than the free drug, in the model of mice bearing P388 leukemia ($p<0.001$, $p<0.05$ and $p<0.05$, respectively). Moreover, one out of 6 mice, in the group treated with F65-MTX conjugate, survived more than 2 months after tumor cells inoculation without any manifestation of leukemia. The results of this experiment are summarized in Table II.

## Discussion

The high-temperature glycation method was used to modify a fibrinogen by D-glucose. Previously, the method had been applied to conjugate different sugars (dextran, lactose, glucose and fructose) with bovine serum albumin, goat anti-rabbit IgG and trypsin. Heating of lyophilized antibodies and enzymes at temperatures above 100˚C did not affect protein activities (11). This was the first time we used the high-temperature glycation method to modify the fibrinogen. We adapted the reaction conditions and examined their influence on the fibrinogen activities. Heating the lyophilized mixture of fibrinogen and D-glucose at various temperatures from 65 to 85˚C resulted in highly substituted conjugates. The level of substitution was dependent on the reaction temperature. The conjugate obtained at 65˚C contained 58 mol glucose/mol fibrinogen, while that obtained at 85˚C, surprisingly, 164 mol glucose/mol fibrinogen.

Modifications of the fibrinogen by D-glucose residues influenced the ability of fibrinogen to clot and its susceptibility to digestion by plasmin. The clottabilities of fibrinogens glycated at 65, 70 and 75˚C were higher than

that of the unmodified protein. These results are consistent with the report of Krantz *et al.*, in which it was shown that glycation carried out in solution increased fibrinogen clottability, explained by increased aggregation of the fibrin monomer. A slightly decreased solubility of the glycated fibrinogens also supported the effect (8). However, in our experiment, the ability of fibrinogens glycated at 80 and 85˚C to clot was either similar to or lower than that of the native protein. This phenomenon could be explained by the very high levels of substitution of these fibrinogens by D-glucose, which probably caused significant conformational changes of fibrinogen molecules. There were also evident differences between the rate of fibrinogenolysis of native fibrinogen and those of glycated fibrinogens. The more substituted the fibrinogen, the more resistant it was to plasmin digestion. The increased resistance of glycated fibrinogen is a known fact, confirmed by a number of laboratories. It is connected with the modifications of lysine amino groups by glucose and some conformational changes of the molecule (8, 9, 19).

The effect of high-temperature glycation on the clottability of fibrinogen and its susceptibility to digestion by plasmin was the result of modifications of ε-amino groups of lysine residues. Heating the lyophilized fibrinogen without glucose did not significantly influence its biological activities. We did not notice any significant differences between the rates of plasmin digestion (data not shown) and the clottabilities of lyophilized fibrinogen and heated fibrinogen.

We compared the usefulness of native and glycated fibrinogen as carriers of the antitumor drug. Fibrinogens glycated at 65 and 73˚C were chosen for coupling with MTX. Our *in vivo* experiment showed that the conjugates of MTX and glycated fibrinogens (F65-MTX and F73-MTX) revealed higher antitumor activity than the free MTX. However, we did not observed any significant superiority of these conjugates over the native fibrinogen-MTX conjugate, with regard to ILS results. It should be pointed out that one long-term survivor was found in the group treated with glycated fibrinogen-MTX conjugate. We expected that the conjugates of glycated fibrinogens and MTX would be present for longer in the proximity of the tumor cells, and that, therefore, they would be more effective than the conjugate of native fibrinogen. It is possible that we did not observe higher activity of these conjugates because, in our model (leukemia), the tumor cells were disseminated in the peritoneum and did not grow in the form of a local tumor. This model seems to be suitable for primary studies of the antitumor activity of the preparations. However, it seems necessary to conduct a series of experiments involving solid tumor models (*e.g.* subcutaneously growing), in which the conjugates are applied systemically (*i.p.* or *i.v.*) or locally (peritumorally). Perhaps such a model would underline the potential and expected differences between conjugates based on unglycated and glycated fibrinogens. It would also be interesting to compare the usefulness of conjugates containing native and glycated fibrinogen applied locally after surgical excision of the tumor. Proteolytic degradation of the conjugates would result in a slow release of the drug in the place of tumor growth. Carrier fibrinogen would additionally act as a sealant, having a positive effect on surgical outcome. Fibrin-based biopolymers are currently used as sealants in a number of surgical procedures. They improve time to hemostasis, reduce blood loss and diminish the frequent complications (20, 21). Intralesional fibrin glue injection has been applied in surgery for cavernous sinus cavernous hemangioma (22). The effect of the sealant can be enhanced by including bioactive agents, *e.g.* antitumor drugs.

It must be emphasized that, in our *in vivo* experiment, most of the mice treated with the conjugates survived longer than mice from the untreated control group. The fibrinogen-MTX conjugates obtained by the previous procedure were toxic at a similar dose (42 mg of MTX per kg of body weight) (4). Decreased toxicity is probably connected with the modifications introduced into the method of MTX activation. In the previous experiments, MTX was treated with NHS in the presence of DCC. This could lead to activation of two carboxylic groups of MTX and enhance the cross-linking of the carrier. To avoid this problem, the procedure of MTX activation was modified. First, the MTX anhydride was prepared in the reaction of MTX with DCC. Then NHS was added to obtain the active ester. We hypothesize that the chemistry of the two-stage activation allows the formation of only mono-activated MTX molecules. The fibrinogen-MTX conjugates prepared by the modified method were more soluble than the conjugates prepared by the previous method.

In conclusion, the MTX conjugates based on glycated fibrinogen did not reveal higher antitumor activity than conjugates based on the native protein in a mouse P388 leukemia model. However, further experiments involving other tumor models are required to elucidate the expected differences.

## Acknowledgements

This work was supported by grant no. 4/2004 from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland.

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*Received January 10, 2005*
*Accepted April 8, 2005*