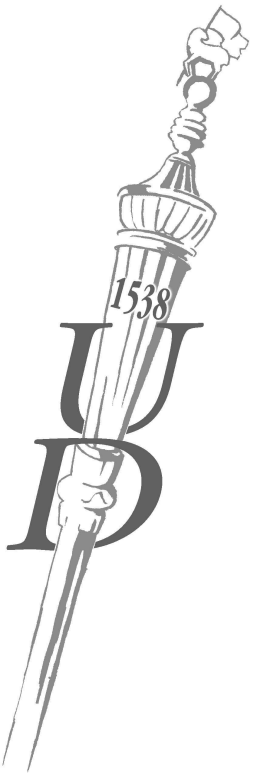
SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Study on factors influencing myelotoxicity of cytotoxic drugs in obese and insulin resistant animal models

by Krisztina Géresi

Supervisor: Ilona Benkő, MD, PhD



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The Examination takes place at the Library of Department of Pharmacology, Faculty of Pharmacy, University of Debrecen; 20th February 2015. at 11:00.

Head of the **Defense Committee**:	Árpád Tósaki, DP, PhD, DSc
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Members of the Defense Committee:	Emese Kiss, MD, PhD, DSc Attila Kiss, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen; 20th February 2015. at 13:00.

1. INTRODUCTION

In our days obesity has become a worldwide epidemic, the incidence of excess body weight and obesity significantly increased during the last two decades both in children and adults. WHO reported that there are 1.1 billion obese and at least 312 million overweight people affected in the world. Obesity leads to many health diseases, such as diabetes mellitus, cardiovascular, pulmonary and metabolic dysfunctions. Moreover evidence shows that obesity is related to increased risk of several types of tumours including colorectal, breast, endometrial, renal, pancreatic, oesophageal and gastric cancers.

Myelosuppression is the most frequent dose-limiting toxicity of chemotherapy during the complex anticancer treatment. This is a consequence of the fact that selectivity of most common anticancer drugs is due to killing primarily the rapidly dividing cells, meaning that high cytotoxic drug concentrations can also damage normal cells with high turnover, especially cells in the bone marrow and gastrointestinal tract. Anticancer agents can inhibit the proliferation of both stem and progenitor cells in bone marrow, but progenitor cells have shorter duplication times than stem cells; therefore, they are more sensitive to chemotherapy-induced damage. Inhibition of proliferation of granulocyte-macrophage progenitor cells (CFU-GM), the common ancestors of granulocytes and macrophages, can cause serious neutropenia with severe consequences, e.g. higher risk for serious infections, which are the leading cause of mortality during chemotherapy of cancer patients.

Our previous work was the first demonstration of myeloprotective effect of rosiglitazone, an insulin-sensitizer drug and we also found that rosiglitazone accelerated recovery of bone marrow damaged by single or repeated doses of 5-fluorouracil possibly by amplifying endogenous insulin action. It draws attention to the possible influence of the insulin resistance on bone marrow functions. As most frequently insulin resistance associates with obesity affects a large growing population.

2. PURPOSES

The main goals of my present research were follows:

To investigate

- whether the femoral hemopoiesis are modified in obese status in physiological conditions or insulin resistance may influence effects of a cytotoxic drug on peripheral blood cell count and bone marrow functions of an obese, insulin resistant animal model
- the effects of three cytotoxic drugs with different mechanism of action on CFU-GM progenitor cells of obese, insulin resistant animals in vitro colony assays
- the effects of pre-treatment of rosiglitazone, an insulin sensitizer drug on hemopoiesis of animals and study whether rosiglitazone can reduce the sensitivity of CFU-GM progenitor cells against cytotoxic drugs
- the effect of UD29 on JY cells (a human leukemic B-cell line), in vitro and in vivo, by determining the decrease of the total cell number and the colony forming activity of the leukemia cells, moreover the effects of UD29 is determine on the colony forming capability of normal CFU-GM progenitors in vitro and in vivo experiments
- the sensitivity of UD29 on CFU-GM progenitor cells of obese, insulin resistant mice compared with sensitivity of carboplatin, doxorubicin and 5-fluorouracil

3. METHODS

3.1. Animals

The experiments conform to the European Community's guiding principles for the care and use of laboratory animals. Studies were carried out according to the approval of the Hungarian National Ethics Committee for Animal Research (1/2009 DEMAB).

Twelve-week-old male control (24.9±2.5g) (C57BLKS/J-*mLep*^{db}/+) and *db/db* (45.2±2.7g) (C57BLKS/J-*mLep*^{db}/*mLep*^{db}) mice were purchased from Janvier Labs (Le Genest Saint Isle, France). We used 19 to 20 week old female mice (23.6±2.4g) (Charles River Laboratories, Budapest, Hungary) with severe combined immunodeficiency (SCID) to transplant human lymphoid B-cells.

Furthermore 11- to 12-week-old Wistar (362±19 g), Zucker (428±24 g) and Goto-Kakizaki (305±13 g) male rats were purchased from Charles River Laboratories (Budapest, Hungary) and were used throughout the experiments.

3.2. Human samples

The bone marrow samples were aspirated from two patients for diagnostic purposes; in the subsequent examinations they did not show any haematological malignancies. The management of patients conformed to the Helsinki Declaration.

3.3. JY cell line

In our studies we used a human leukemic B cell line (JY) for in vitro and in vivo investigations using serious immunodeficient (SCID) mice, in which human tumoral growth can be examined.

3.4. Bone marrow samples

- Bone marrow samples from human

Human bone marrow samples were aspirated for diagnostic purposes. Heparin (100 U/mL) was added to the samples. After the cell aggregates had been dispersed bone marrow was mixed with McCoy’s 5A medium at 1:1 (v/v) ratio. The mononuclear cell fraction was separated by Ficoll gradient centrifugation (1.077 g/ml) at 1000g for 15 minutes. Buffy coat cells were washed twice with McCoy’s 5A medium.

- Bone marrow samples from mice

Bone marrow was obtained from the femurs of the mice after their extermination by cervical dislocation. Femurs were removed and the bone marrow cells were aseptically washed out.

- Bone marrow samples from rats

Animals were exterminated by intravenous overdose of thiopental sodium. Femoral bones of rats were prepared under sterile conditions and bone marrow was completely washed out. Single cell suspensions were obtained by suspending bone marrow samples in McCoy's 5A medium and forcing them several times through a thin needle with a syringe. We separated the mononuclear cell fractions from bone marrow cell suspensions by Ficoll gradient centrifugation (1.077 g/ml) at 1000g for 15 minutes. Buffy coat cells were washed twice with McCoy's 5A medium.

3.5. Obese, insulin resistant animal models

Db/db mouse is an accepted competent model of human obesity and insulin resistance, because of a spontaneous autosomal recessive mutation on chromosome 4 that inhibits the expression of leptin receptors. The developing leptin resistance causes obesity and diabetes mellitus type 2.

Zucker rats are competent models of an obese and insulin resistant status, because of a spontaneous obesity-causing mutation in the leptin receptor gene, an autosomal recessive mutation on chromosome 5. They exhibit obesity at 4–5 weeks of age, and have many metabolic characteristics in common with human obesity-associated type-2 diabetes such as insulin resistance, hyperlipidemia, hypercholesterolemia and hyperinsulinemia. Goto-Kakizaki rats are well characterized models of non-insulin dependent diabetes mellitus. They are non-obese but insulin resistant animals showing genetically determined alterations of glucose tolerance and insulin secretion. In our experiments Wistar rats were used as a non-obese and non-insulin resistant control.

3.5.1. Characteristics of hemopoiesis of insulin resistant animal models compared with control groups in the absence of cytotoxic drugs

First in 10 control and 10 *db/db* mice after 10 Zucker rats, 10 Wistar rats and 10 Goto-Kakizaki rats we determined the cellularity, the total nucleated cell number of the femoral

bone marrow, which mirrors hemopoietic activity. Then the common progenitors of the phagocytic cells, the granulocyte-macrophage colony forming units were studied and compared with the hemopoiesis of control rats. The CFU-GM progenitors were cultured in a specific soft gel colony assay. The frequency of CFU-GM progenitors was established from these soft gel cultures. Total CFU-GM content of the femur was calculated from cellularity and frequency of CFU-GM progenitor cells.

3.5.2. In vivo effect of carboplatin on peripheral blood cell count and bone marrow function in control and db/db mice

Myelopoiesis and peripheral blood cell counts were studied after in vivo intraperitoneal administration of a single high dose carboplatin (100 mg/kg) in 10 control and 10 obese *db/db* mice. Peripheral white blood cell count, femoral cellularity, frequency of CFU-GM progenitor cells and total femoral CFU-GM content were determined 48 hours following the injection of carboplatin.

3.5.3. In vitro sensitivity of granulocyte-macrophage progenitor cells (CFU-GM) of control and obese animals to carboplatin, doxorubicin and 5-fluorouracil

10 -10 control and *db/db* mice, furthermore 10 Wistar, 10 Zucker and 10 Goto-Kakizaki were exterminated to study the direct effects of cytotoxic drugs on CFU-GM progenitor cells, nucleated bone marrow cells were cultured in vitro in the presence of increasing concentrations (0.001-10 mg/L) of either carboplatin or doxorubicin or 5-fluorouracil. These three anticancer drugs with different mode of actions were selected intentionally to check for any potential drug specific effect on the sensitivity of CFU-GM progenitor cells.

3.5.4. Effect of rosiglitazone pre-treatment on insulin sensitivity of Goto-Kakizaki rats

We pre-treated 8 Goto-Kakizaki rats with rosiglitazone, an insulin sensitizer drug, using 3 mg/kg daily doses *in vivo*. Rosiglitazone or its vehicle was administered orally to animals on fourteen consecutive days. After the pre-treatment period animals were anesthetized with an initial intraperitoneal dose of 50 mg/kg thiopental-sodium that was repeated as needed. Insulin sensitivity was determined by rapid insulin sensitivity test (RIST).

3.5.5. Effect of rosiglitazone pre-treatment on sensitivity of CFU-GM progenitors to cytotoxic agents in groups of pre-treated Goto-Kakizaki rats

After rosiglitazone pre-treatment we studied the viability of CFU-GM progenitor cells of pre-treated Goto-Kakizaki rats using in vitro colony assays, in which CFU-GM cells were grown in the presence of carboplatin or doxorubicin or 5-fluorouracil in the same dose ranges as in bone marrow cultures of obese Zucker and vehicle-treated Goto-Kakizaki rats.

3.5.6. Effect of rosiglitazone pre-treatment on sensitivity of CFU-GM progenitors to carboplatin, doxorubicin and 5-fluorouracil in groups of pre-treated Wistar rats

At the end of the rosiglitazone pre-treatment we studied the sensitivity of CFU-GM progenitor cells of pre-treated Wistar rats using in vitro colony assays, in which CFU-GM cells were grown in the presence of carboplatin or doxorubicin or 5-fluorouracil in the same dose ranges as in bone marrow cultures of obese Zucker and Goto-Kakizaki rats.

3.6. Effects of UD29 (4-thio-uridine-monophosphate) on leukemia stem cells and normal CFU-GM progenitor cells in vitro and in vivo experiments

3.6.1. Leukemia animal model

Forty SCID mice were randomly assigned into two groups first. 20 mice were implanted with 2x 10^7 JY cells intravenously and vehicles were administered to the other group of mice. Then the groups were further divided into two parts. Mice in the two control groups (transplanted and not transplanted) were given vehicle and the other two groups were treated with UD29 intraperitoneally from the 3rd day of post-transplantation. Treatment schedule included repeated administration of UD29 in 250 mg/kg doses every second day. At the end of this protocol mice were exterminated by cervical dislocation and their femoral bone marrow were tested. After the intravenous transplantation of JY cells, bone marrow was infested by leukemic tumor cells. The effects of UD29 on the bone marrow cells were measured by the change of total cell content in the femur, the change of total femoral leukemic stem cell content and the change of the frequency of leukemic stem cells. The effect of UD29 in non-transplanted SCID mice was studied with similar methods as in transplanted animals determining the total cell content and the total granulocyte–macrophage progenitor cells in femoral bone marrow.

3.6.2. Antiproliferative effect of UD29 on JY cell line in vitro colony assays

Using 35 mm plastic petri dishes, 1 x 10^4 /mL B-lymphoma cells were plated and were incubated for 7 days at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2 supplemented with 20% foetal bovine serum and methylcellulose without any growth factors.

Cultures were grown in the presence of UD29 in increasing concentration. Cultures were grown in triplicates.

3.6.3. Effect of UD29 on normal granulocyte-macrophage progenitor cells of human bone marrow samples

The colony forming activity of normal CFU-GM progenitor cells in the presence of UD29 was determined. Cultures were contained UD29 in increasing concentration. Cells were incubated for 14 days at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2 supplemented with 20% foetal bovine serum and methylcellulose, medium was supplemented with colony stimulated factors. Cultures were grown in triplicates.

3.6.4. Effect of UD29 on colony forming capability of CFU-GM progenitor cells of obese, insulin resistant db/db mice

After the extermination of 10 controls and 10 *db/db* mice, femoral bone marrow was aseptically removed then colony assays were made. We tested the sensitivity of CFU-GM progenitor cells of obese, insulin resistant *db/db* mice in the presence of UD29.

3.7. Statistical analysis

Statistical analyses were performed by GraphPad Prism Software (GraphPad Software, Inc. La Jolla, CA, USA). Data obtained from individual mice were used for statistical analysis and each variable was evaluated by comparing the obese and non-obese groups with Student's unpaired t test. Differences were regarded as statistically significant at $p<0.05$.

Data obtained from individual rats were used for statistical analysis. Each variable was evaluated using one-way analysis of variance, followed by Bonferroni's post-test for multiple comparisons. Differences were regarded as statistically significant at $p<0.05$.

In dose-response curves fit to the 4-parameter logistic curve-variable slope.

4. RESULTS

4.1. Characteristic of hemopoiesis of obese, insulin resistant animals and their controls

Bone marrow function was evaluated by total cellularity, frequency of granulocyte-macrophage progenitors and total CFU-GM content of the femoral bone marrow. Total cellularity of femoral bone marrow reflects the intensity of hemopoiesis. No differences were demonstrated in total femoral cellularity between control and obese *db/db* mice furthermore we did not demonstrate differences in total femoral cellularity in control Wistar and obese, insulin resistant Zucker rats and non-obese, insulin resistant Goto-Kakizaki rats.

Frequency of CFU-GM progenitors was estimated from soft gel cultures. In these cultures the descendant cells of the proliferating CFU-GM progenitors remain together and form colonies. Numbers of colonies grown from mononuclear bone marrow cells show the frequency of progenitors indicating the intensity of granulopoiesis in bone marrow. There were no alterations in number of CFU-GM progenitors in *db/db* mice compared to control mice. Moreover we did not find alterations in number of CFU-GM progenitors in Zucker and Goto-Kakizaki rats compared with Wistar rats. Colony forming capability of CFU-GM progenitors of obese insulin resistant animals was not impaired in the absence of cytotoxic drugs. According to this, hemopoiesis seemed to be intact in these obese, diabetic animals.

4.2. In vivo effect of carboplatin on peripheral blood cell counts and bone marrow function in control and db/db mice

White blood cell counts were significantly lower in obese *db/db* mice than in control mice 48 hours after a single 100 mg/kg intraperitoneal dose of carboplatin. This decrease was primarily due to decreased number of circulating neutrophil granulocytes. Absolute neutrophil counts dropped into the very low range ($0.63\pm0.10 \times 10^9$/L vs $3.32\pm0.18 \times 10^9$/L in untreated controls). In carboplatin treated control mice the absolute neutrophil count was twice as high as in carboplatin treated *db/db* mice ($1.35\pm0.09 \times 10^9$/L).

A single 100 mg/kg dose of carboplatin resulted in serious bone marrow damage. The decrease in CFU-GM progenitor cells was evident 48 hours after carboplatin administration. Carboplatin significantly decreased the cellularity and the frequency of CFU-GM progenitors in groups of *db/db* mice compared to non-obese mice. As a consequence the total CFU-GM pool became significantly lower which means less regenerative capacity to restore bone marrow functions after the damage. Carboplatin caused more serious damage in bone marrow function in obese *db/db* mice than in their littermates.

4.3. In vitro sensitivity of granulocyte-macrophage progenitor cells (CFU-GM) of control and obese animal models to carboplatin, doxorubicin, 5-fluorouracil

The sensitivity of CFU-GM, isolated from control and *db/db* mouse, Wistar, Zucker and Goto-Kakizaki rats was studied in vitro. In the presence of increasing concentrations of cytotoxic drugs a dose dependent decrease in CFU-GM numbers were observed in bone marrow cell cultures of control groups and obese, diabetic animals and non-obese but insulin resistant Goto-Kakizaki rats. However, the sensitivity of CFU-GM cells of obese animals and Goto-Kakizaki rats was increased to cytotoxic drugs. Colony numbers were significantly lower for each investigated cytotoxic drugs - carboplatin, doxorubicin and 5-fluorouracil. These drugs were more toxic to CFU-GM progenitors of *db/db* mice, Zucker and Goto-Kakizaki rats compared to their littermates.

4.4. Effect of rosiglitazone pre-treatment on insulin sensitivity and sensitivity of CFU-GM progenitors to cytotoxic agents in groups of pre-treated Goto-Kakizaki and Wistar rats

We pre-treated Goto-Kakizaki rats with rosiglitazone, an insulin sensitizer drug. Rosiglitazone was administered orally at a daily dose of 3 mg/kg body weight for fourteen days in vivo. This pre-treatment resulted in significantly higher insulin sensitivity compared with vehicle-treated Goto-Kakizaki rats' insulin sensitivity. After rosiglitazone pre-treatment insulin sensitivity increased and it was not significantly different from insulin sensitivity of control Wistar rats.

After rosiglitazone pre-treatment we could establish increased viability of CFU-GM progenitor cells using in vitro colony assays, in which CFU-GM cells were grown in the presence of carboplatin or doxorubicin or 5-fluorouracil in the same dose ranges as in bone marrow cultures of obese Zucker and vehicle-treated Goto-Kakizaki rats. Effect of rosiglitazone on vulnerability of CFU-GM progenitor cells were decreased to carboplatin, doxorubicin and 5-fluorouracil in rosiglitazone pre-treated groups compared with vehicle-treated Goto-Kakizaki rats. By rosiglitazone pre-treatment we could improve the insulin sensitivity and concurrently decrease the toxicity of cytotoxic drugs on CFU-GM progenitor cells of Goto-Kakizaki rats.

4.5. Effect of rosiglitazone pre-treatment on sensitivity of CFU-GM progenitors to cytotoxic agents in groups of pre-treated Wistar rats

Some increased viability of CFU-GM progenitors was observed to the studied cytotoxic drugs also in the rosiglitazone pre-treated control Wistar rats however it was not significant in majority of cases. Similarly rosiglitazone had no significant effect on the healthy bone marrow in 5-day-long treatment in vivo in our previous experiments.

4.6. Results of investigations of 4-thio-uridine-monophosphate (UD29)

4.6.1. Effect of UD29 on colony formation of tumor stem cells and healthy human bone marrow granulocyte-macrophage progenitor cells in vitro

UD29 decreased tumor stem cell colony numbers of the JY cell line in vitro dose-dependently. We examined the mononucleotide's effects on CFU-GM progenitors of healthy human bone marrow. UD29 did not block colony formation of human normal bone marrow CFU-GM progenitor cells significantly even at a higher concentration. It means that UD29 does not cause neutropenia, which is the most feared side effect of cytotoxic anticancer drugs.

4.6.2. In vivo effects of UD29 on hemopoiesis in JY transplanted and JY not-transplanted groups of SCID mice

In vivo the SCID mice serve one of the best experimental model for human tumor growing. These mice would accept human tumor xenografts and reflect clinical results with regard to response to chemotherapy. JY human B leukemic cells were transplanted to SCID mice and vehicle or UD29 were administered them intraperitoneally. Significantly decreased tumor cell content was observed in tumor-infested bone marrow of the JY transplanted SCID mice in the UD29-treated groups. Contrarily UD29 did not change the cellularity of normal bone marrow in SCID mice without transfection with tumor cells. After the UD29's treatments the number of the colony-forming tumor stem cells was also decreased significantly in leukemic SCID mouse groups. UD29 decreased nor CFU-GM colony numbers, neither CFU-GM content in tumor-free, not transplanted groups.

4.6.3. In vitro sensitivity of granulocyte-macrophage progenitor cells (CFU-GM) of control and obese db/db mice to 4-thiouridine-monophosphate

Four-thiouridine-monophosphate, analogously to the cytotoxic drugs, resulted in a dose dependent decrease in CFU-GM colony numbers in bone marrow cell cultures of both

control and *db/db* mice and, also correspondingly to the anticancer drugs, the sensitivity of CFU-GM cells of obese *db/db* mice was higher to UD29.

Doxorubicin was the most, and carboplatin was the least toxic to the CFU-GM progenitor cells among the studied common cytotoxic drugs. Four-thiouridine-monophosphate, an anticancer drug under investigation, was much less toxic to CFU-GM cells. In addition, the differences were higher in diabetic *db/db* mice. The fifty percent inhibitory concentration of UD29 on CFU-GM cells from *db/db* mice was 25 times higher than carboplatin and 6500 times higher than doxorubicin, while in the control mice the same differences were 15 and 4100 times, respectively.

5. DISCUSSION

The aims of our investigations we studied whether the femoral hemopoiesis and function of femoral CFU-GM progenitor cells are modified in obese status or insulin resistance and may influence effects of cytotoxic drugs on bone marrow.

A chemically modified mononucleotide, 4-thio-uridine-monophosphate was shown to have anti-HIV and anti-proliferative activity on tumor and leukemic cell lines. We determined the effect of UD29 on JY cells, in vitro and in vivo, by determining the decrease of the total cell number and the colony forming activity of the leukemia cells. We also tested the toxicity of UD29 on normal human CFU-GM progenitor cells in vitro and in vivo experiments we determined the cellularity of normal bone marrow, the colony forming capability of CFU-GM progenitors and the total femoral CFU-GM content in SCID mice without transplantation with tumor cells.

Bone marrow function was evaluated by total cellularity, frequency of CFU-GM progenitors and total CFU-GM content of the femoral bone marrow. Total cellularity of femoral bone marrow reflects the intensity of hemopoiesis. No differences were demonstrated in total femoral cellularity between control and obese *db/db* mice and in our further investigations we did not demonstrate differences in total femoral cellularity in control Wistar and obese, insulin resistant Zucker rats and non-obese, insulin resistant Goto-Kakizaki rats.

Frequency of CFU-GM progenitors was estimated from soft gel cultures. In these cultures the descendant cells of the proliferating CFU-GM progenitors remain together and form colonies. Numbers of colonies grown from mononuclear bone marrow cells show the frequency of progenitors indicating the intensity of granulopoiesis in bone marrow. There were no alterations in number of CFU-GM progenitors in *db/db* mice compared to control

mice. Furthermore we did not find alterations in number of CFU-GM progenitors in Zucker and Goto-Kakizaki rats compared with Wistar rats. Colony forming capability of CFU-GM progenitors of obese, insulin resistant mice was not impaired in the absence of cytotoxic drugs. According to this, hemopoiesis seemed to be intact in these obese, diabetic animals.
Nevertheless, after in vivo administration of a high dose of carboplatin, cellularity of bone marrow, frequency and total femoral content of CFU-GM progenitors were significantly decreased in *db/db* mice than in the control animals. The increased myelotoxicity, at least in part, seemed to be a direct effect on myeloid progenitors since an increased in vitro sensitivity of CFU-GM progenitors of *db/db* mice was found by culturing them in the presence of three established cytotoxic drugs with different mechanism of actions (carboplatin, doxorubicin and 5-fluorouracil). In the presence of increasing concentrations of cytotoxic drugs a dose dependent decrease in CFU-GM numbers were observed in bone marrow cell cultures of control groups and obese, diabetic mice. However, the sensitivity of CFU-GM cells of *db/db* mice was increased to cytotoxic drugs. Colony numbers were significantly lower for each investigated cytotoxic drugs - carboplatin, doxorubicin and 5-fluorouracil. These drugs were more toxic to CFU-GM progenitors of *db/db* mice compared to their littermates.

In further investigations we found increased sensitivity of CFU-GM progenitor cells of obese, insulin resistant Zucker rats to cytotoxic drugs was found by culturing them *in vitro* in the presence of carboplatin, doxorubicin and 5-fluorouracil. All drugs were more toxic on CFU-GM progenitor cells of Zucker rats. This might be based on metabolic disorders, at least in part, because we could demonstrate a similar increase in toxicity of the studied anticancer drugs to the CFU-GM progenitors originated from Goto-Kakizaki rats in the same dose ranges. After in vivo administration of rosiglitazone, an insulin sensitizer, the anticancer drug sensitivity of CFU-GM progenitors of Goto-Kakizaki rats was decreased concurrently with improvement of insulin resistance.

Slightly increased viability of CFU-GM progenitors to the studied cytotoxic drugs was also observed in the rosiglitazone pre-treated control Wistar rats however it was not significant in the majority of cases. Similarly rosiglitazone had no significant effect on the healthy bone marrow in 5-day-long treatment in vivo in our previous experiments and insulin had no effect on the control healthy mice using the same administration schedule, too. By rosiglitazone pre-treatment we could improve the insulin sensitivity and concurrently decrease the toxicity of cytotoxic drugs on CFU-GM progenitor cells of Goto-Kakizaki rats. The dose-response curves of the studied cytotoxic drugs were shifted to the normal ranges after

rosiglitazone pre-treatment, however they do not completely reached the values of the control Wistar and rosiglitazone pre-treated Wistar rats in the case of doxorubicin and 5-fluorouracil.

Henceforward we tested the sensitivity of the femoral CFU-GM progenitor cells to 4-thiouridylate which showed anti-proliferative activity on various tumor cell lines and we observed its antiproliferative effect both in vitro and in vivo in SCID mice against JY cells, a model for human acute lymphoid leukaemia. Thiouridilate decreased tumor stem cell colony numbers of the JY cell line in vitro. In a parallel experiment UD29 could not block colony formation of human normal bone marrow CFU-GM progenitor cells significantly even at a high concentration. In the in vivo study both the total cell content and the total colony forming activity of the femur decreased significantly. However, the ratio of the total cell number to the total colony forming activity in the femur did not change on the treatment suggesting that both mature leukemia cells and leukemia stem cells were equally sensitive to the nucleotide. When healthy, untransplanted animals were treated with s4UMP neither the total cell number, nor the colony forming activity was changed indicating the nontoxic nature of the compound on hematopoietic precursor cells.

Since 4-thiouridylate, as a nucleotide, cannot penetrate into the cells, it must exert its activity at the cell surface. The enol-form of the compound carries a reactive –SH group which might interact with the cysteinyl side chains of proteins affecting their biological function. It suggested that 4-thiouridylate exerts its anti-proliferative activity by affecting thiol/disulfide exchange processes of cell surface proteins, including receptors, involved in the regulation of cell cycle or cell death. The pattern of DNA degradation and the elevated caspase-9 activity with characteristic changes in the morphology of treated cells strongly suggested that 4-thiouridylate induces apoptosis.

6. NEW FINDINGS

The most significant aspect of our new results is to emphasize dysfunction of haemopoietic progenitor cells in obesity, diabetes and insulin resistance. This dysfunction could not be detected under physiologic circumstances. However, the vulnerability of CFU-GMs (the common progenitor cells of monocytes, granulocytes and macrophages) to cytotoxic drugs was increased both in vivo and in vitro. While the increased in vivo vulnerability might be due to not only direct effects of drugs on progenitor cells but also to variations in drug exposure (resulting from pharmacokinetic differences) or to effects on other cells in the bone marrow, the increased in vitro vulnerability directly demonstrates the damage of progenitor cells. Progenitor cells were more vulnerable to effects of cytotoxic drugs independently of their mechanism of actions or the localization of their target molecules (intracellular or cell membrane). Disorders in cell metabolism and receptorial functions may be responsible for the increased vulnerability which is possibly aggravated by modified cross-talk among bone marrow cells and the dysfunctional bone marrow stroma.

In clinical practice early and low absolute neutrophil count is the most important sign of serious myelotoxicity and this immunosuppressed period might lead to development of life-threatening infections with high mortality. The longer duration of regeneration may result in delay of the next chemotherapy course leading to reduced antitumor efficacy and survival. Our results warn that weight loss and normalization of glucose homeostasis may be important before cytotoxic chemotherapy in patients with obesity and DMT2 and might improve the outcome of chemotherapy of malignant diseases.

Consequently the investigated UD29 has no effect on normal granulocyte-macrophage progenitor cells. Thiouridilate showed selective effect on leukemic stem cells both in vitro and in vivo. It has an importance as an anticancer candidate molecule, which has to kill tumor stem cells to prevent the quick tumor re-growth supported by this very aggressive tumor cell subpopulation, which is present only in about 0,1-2% ratio among the tumor cells.

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I extremely grateful to my Family, they have always given support and courage to me throughout my whole academic career.

8. APPENDIX

UNIVERSITY OF DEBRECEN
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PUBLICATIONS

Register number: DEENK/5/2015. PL
Item number:
Subject: Ph.D. List of Publications

Candidate: Krisztina Géresi
Neptun ID: HSX71I
Doctoral School: Doctoral School of Pharmaceutical Sciences
Mtmt ID: 10047765

List of publications related to the dissertation

1. **Géresi, K.**, Megyeri, A., Szabó, B., Szabó, Z., Aradi, J., Németh, J., Benkő, I.: Myelotoxicity of carboplatin is increased in vivo in db/db mice, the animal model of obesity-associated diabetes mellitus.
   Cancer Chemother. Pharmacol. "Accepted by Publisher" (2015)
   IF:2.571 (2013)

2. **Géresi, K.**, Benkő, K., Szabó, B., Megyeri, A., Peitl, B., Szilvássy, Z., Benkő, I.: Toxicity of cytotoxic agents to granulocyte-macrophage progenitors is increased in obese Zucker and non-obese but insulin resistant Goto-Kakizaki rats.
   Eur. J. Pharmacol. 696 (1-3), 172-178, 2012.
   DOI: http://dx.doi.org/10.1016/j.ejphar.2012.09.018
   IF:2.592

3. Berényi, E., Benkő, I., Vámosi, G., **Géresi, K.**, Tárkányi, I., Szegedi, I., Lukács, L., Juhász, I., Kiss, C., Fésüs, L., Aradi, J.: In vitro and in vivo activity of 4-thio-uridylate against JY cells, a model for human acute lymphoid leukemia.
   Biochem. Biophys. Res. Commun. 410 (3), 682-687, 2011.
   DOI: http://dx.doi.org/10.1016/j.bbrc.2011.06.056
   IF:2.484

List of other publications

4. Miszti-Blasius, K., Felszeghy, S., Kiss, C., Benkő, I., **Géresi, K.**, Megyeri, A., Hevessy, Z., Kappelmayer, J.: P-selectin glycoprotein ligand-1 deficiency augments G-CSF induced myeloid cell mobilization.
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   IF:1.266

Total IF of journals (all publications): 11,273
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List of posters related to the dissertation:

1. **Géresi K.**, Szegedi I., Megyeri A., Aradi J., Kiss Cs., Benkő I.
Thiouridilát tumor ellenes hatásának *in vitro* és *in vivo* vizsgálata.
The 9th Conference of the Hungarian Association for Clinical and Experimental Pharmacology, Debrecen,2007.

2. **Géresi K.**, Szegedi I., Megyeri A., Aradi J., Kiss Cs., Benkő I.
Effect of thiouridilate (UD29) on colony forming capability of leukemia tumor stem cells (*in vitro* and *in vivo* investigations).
Conference of Ph. D. students in Sanofi-Aventis/Chinoin, 2008.

3. **Géresi K.**, Szegedi I., Megyeri A., Aradi J., Kiss Cs., Benkő I.
Effect of thiouridilate (UD29) on colony forming capability of leukemic tumor stem cells (*in vitro* and *in vivo* investigations).
1st Central and Eastern European Laboratory Animal Conference Budapest, 2009.

4. **Géresi K.**, Benkő K., Megyeri A., Szabó B., Ungvári É., Peitl B., Szilvássy Z., Benkő I.
Toxicity of cytostatic agents to granulocyte-macrophage progenitors (CFU-GM) increased in Zucker obese rats.
WorldPharma (16th IUPHAR World Congress of Basis and Clinical Pharmacology), Copenhagen, Denmark, 2010.

List of presentations related to the dissertation:

1. **Géresi K**., Szegedi I., Megyeri A. Aradi J., Kiss Cs., Benkő I.
Effect of thiouridilate (UD29) on the colony forming capability of leukemia tumour stem cells (in vitro and in vivo investigations).
Conference on The Pharmacology of Immunopharmacons and Biological Agents˝Debrecen,2007.

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3. **Géresi K.**, Benkő K., Megyeri A., Szabó B., Ungvári É., Peitl B., Szilvássy Z., Benkő I.
Study on factors influencing myelotoxicity of cytotoxic drugs in obese animal models.
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