

Myelotoxicity of carboplatin is increased in vivo in db/db mice, the animal model of obesity-associated diabetes mellitus

Journal:	<i>Cancer Chemotherapy and Pharmacology</i>
Manuscript ID:	CCP-14-0618.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Géresi, Krisztina; University of Debrecen, Faculty of Medicine, Department of Pharmacology and Pharmacotherapy Megyeri, Attila; University of Debrecen, Faculty of Medicine, Department of Pharmacology and Pharmacotherapy Szabó, Boglárka; Semmelweis University, Heart and Vascular Center Szabó, Zsolt; CFP AG, Department of Mathematics and Statistics Aradi, János; University of Debrecen, Faculty of Medicine, Department of Biochemistry and Molecular Biology Németh, József; University of Debrecen, Faculty of Medicine, Department of Pharmacology and Pharmacotherapy Benkő, Ilona; University of Debrecen, Faculty of Medicine, Department of Pharmacology and Pharmacotherapy
Keywords:	myelotoxicity, obesity, diabetes mellitus, granulocyte-macrophage progenitor cells CFU-GM, carboplatin, doxorubicin, 5-fluorouracil

Title page**Original Article****Authors:**

Krisztina Géresi¹, Attila Megyeri¹, Boglárka Szabó², Zsolt Szabó³, János Aradi⁴, József Németh¹, Ilona Benkő¹

Title:

Myelotoxicity of carboplatin is increased *in vivo* in *db/db* mice, the animal model of obesity-associated diabetes mellitus

¹University of Debrecen, Faculty of Medicine, Department of Pharmacology and Pharmacotherapy, Nagyerdei blv. 98., H-4032 Debrecen, Hungary

²Semmelweis University, Heart and Vascular Center, Gaál József 9-11., H-1122 Budapest, Hungary

³CFP AG, Department of Mathematics and Statistics, Seestrasse 29., Zollikon, ZH, CH, 8702 Switzerland

⁴University of Debrecen, Faculty of Medicine, Department of Biochemistry and Molecular Biology, Nagyerdei blv. 98., H-4032 Debrecen, Hungary

Corresponding author:

Ilona Benkő M.D., Ph.D.

Department of Pharmacology and Pharmacotherapy

Faculty of Medicine, University of Debrecen,

Nagyerdei krt. 98., H-4032 Debrecen, Hungary

Tel: +36 52 427899; Fax: +36 52 427899; Mobile: +36 20 5463838

E-mail address: ibenko@med.unideb.hu

Abstract

Purpose Some authors observed increased carboplatin-associated myelotoxicity in obese patients which was exclusively attributed to elevated AUC. To investigate the potential contribution of functional changes of cells primarily responsible for myelopoiesis, granulocyte-macrophage progenitors (CFU-GM) were studied in obesity-associated diabetes mellitus (DMT2).

Methods The most frequently used animal model of human obesity with DMT2 is *db/db* mouse. Cellularity, frequency of CFU-GM and total CFU-GM content of femoral bone marrow was measured after 100 mg/kg dose of carboplatin *in vivo*. To exclude influence of pharmacokinetic changes direct toxicity of carboplatin on CFU-GM was also determined *in vitro* and was compared with other anticancer agents, namely doxorubicin, 5-fluorouracil and 4-thiouridylate.

Results After intraperitoneal administration of carboplatin, each measured characteristics of bone marrow function were more significantly suppressed and the induced neutropenia were more serious in *db/db* mice than in the controls. The increased myelotoxicity seemed to be a direct effect on myeloid progenitor cells since their increased *in vitro* sensitivity was found in *db/db* mice. This was not specific for carboplatin, a similar double to 5-fold increase in myelotoxicity of each cytotoxic drug with different mechanism of action was observed. Four-thiouridylate, a promising antileukemic molecule with good therapeutic index, was by far the least toxic for CFU-GM of *db/db* mice.

Conclusions A serious disorder of CFU-GM progenitors was suggested in obese mice with DMT2, which eventually might lead to more severe myelotoxicity and neutropenia. Weight loss and normalization of glucose homeostasis may be important before chemotherapy of malignant diseases in obesity with DMT2.

Keywords: myelotoxicity, obesity, diabetes mellitus, granulocyte-macrophage progenitor cells (CFU-GM), carboplatin, doxorubicin, 5-fluorouracil

Introduction

In recent years obesity has become one of the most threatening public health problems worldwide. It definitely plays a role in the pathogenesis of cardiovascular diseases, but it is also an important risk factor of many types of malignant tumors, including colon, endometrium, breast, kidney, oesophagus, pancreas, gallbladder, liver, and haematological malignancies. Additionally, prognosis of malignancies can also be worse in obese than in non-obese patients [1].

The poorer prognosis in obese patients may be due to alterations of several variables such as tumour behaviour, immune responses or effects of cytotoxic drugs. Regarding drug effects, an inferior treatment outcome might be caused either by a decrease in antitumor efficacy or by an increase in toxicity. Fear from increased therapy-related toxicities influences chemotherapy dosing, usually by applying various formulas to reduce doses calculated by body weights or body surface areas. However, dose reductions may increase the risk of relapses and reduce progression-free or overall survival.

Increased myelosuppression, which is the most frequent dose-limiting toxicity of chemotherapy might be explained by increased drug exposure due to changes in pharmacokinetics of cytotoxic drugs. Indeed, excess accumulation of adipose tissue in obesity may result in substantial variation in pharmacokinetics of some drugs [2]. However, the available pharmacokinetic data in obese patients about most anticancer drugs from properly powered clinical trials are scarce [3]. This paucity of reliable data, at least in part, ascribed to

the fact that drug development and clinical trials in oncology are generally conducted irrespective of patients' body weight, and obesity is a covariate not usually stratified in data analysis. Still, it is generally acknowledged that there are considerable alterations in the pharmacokinetics of some cytotoxic drugs in obesity, which might even lead to increased toxicity but the available data are somewhat contradictory [4-6]. Thompson et al. [7] found no differences in pharmacokinetics of daunorubicin between obese and non-obese patients while obesity worsen outcome of patients with breast cancer on doxorubicin (chemical structure is very close to daunorubicin) + cyclophosphamide therapy [8]. According to the current guidelines, overall, there is no rationale to adjust the standard dosing regimens – based on full weight – in obese patients [3].

Carboplatin is used in many solid tumors, among them those, which correlate well with obesity, e.g. ovarian or breast cancer. Carboplatin elimination is primarily dependent on glomerular filtration rate (GFR), which means that estimation of GFR is essential for optimal dose calculation. The most frequently used Calvert formula, which calculates the dose necessary for achieving an optimal target AUC, is also based on the accurate estimation of the GFR. Several equations have been used to determine the GFR by creatinine clearance. However, as these equations are based on estimation serum creatinine level, they tend to overestimate creatinine clearance in obese patients [9]. The Cockcroft-Gault equation, which is the most frequently used to estimate creatinine clearance in Calvert formula, is no exception. Thus the overestimation of creatinine clearance may result in overdose and higher than expected, extremely high AUC levels in obese patients [4]. To avoid the overdose of carboplatin, dose reduction has been suggested by replacing actual body weight with ideal or adjusted body weights in these calculations [10].

Due to dose reductions lower risk of therapy-related toxicities was observed that obese patients receive suboptimal doses and anticancer therapy may become insufficient [11]. On

contrary even the carboplatin dose was calculated on actual body weight, some authors found worse response to carboplatin-paclitaxel chemotherapy in obese and overweight women with breast or advanced ovarian cancer [12-13]. On the other hand frequency of serious Grade 4 neutropenia, as a consequence of myelotoxicity, was higher in obese than in non-obese women with lung cancer treated by carboplatin and paclitaxel despite appropriate dose reductions [14]. This observation raises possibility of increased sensitivity of the myelopoiesis in obese patients, as the risk of other serious adverse effects was not increased.

Indeed, it is also accepted that obesity may have an effect on myelotoxicity of anticancer treatment not only through pharmacokinetic but also through pharmacodynamic mechanisms [3, 15]. One of the best characterized and most frequent metabolic dysfunction in obesity is insulin resistance which is also a characteristic of diabetes mellitus type 2 (DMT2). In our previous work we found that rosiglitazone, an insulin sensitizer, had myeloprotective effect and ameliorated myelotoxicity of 5-fluorouracil in mice [16-18]. This suggested that insulin resistance may influence bone marrow functions and may alter therapy-related toxicity of anticancer drugs. Later, we were the first to observe an increased *in vitro* toxicity of cytotoxic drugs to the granulocyte-macrophage progenitors in Zucker obese rats with insulin resistance and also in non-obese but insulin resistant Goto-Kakizaki rats [15]. The association of increased *in vitro* sensitivity of CFU-GMs with obesity and/or insulin resistance suggested a direct damage of these progenitor cells in obesity. Only these few data can be found in the current scientific literature about changes caused by obesity in target cells of bone marrow and their influence on chemotherapy.

Carboplatin is frequently used in anticancer protocols in ovarian, breast, testicular and prostate cancer, which are strongly associated with obesity. The aims of our current studies was to evaluate myelotoxicity of carboplatin *in vivo* in obesity-associated diabetes, in which metabolic and non-metabolic influences may be stronger on bone marrow cells than in insulin

resistance or diabetes mellitus without obesity and may have the highest importance in clinical practice. We focused on the main target, the granulocyte-macrophage progenitors (CFU-GM), in obesity-associated diabetes mellitus type 2 (DMT2) to assess, whether their functional changes may additionally result in increased myelotoxicity.

Methods

Animals

Twelve-week-old male control (C57BLKS/J-*mLep*^{db/+}) and *db/db* (C57BLKS/J-*mLep*^{db/mLep}^{db}) mice were purchased from Janvier Labs (Le Genest Saint Isle, France). The present 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).

Chemicals

Following drugs were used: carboplatin (Paraplatin, Bristol Myers-Squibb, Sermoneta, Italy), doxorubicin (Adriblastina, Pharmacia & Upjohn SPA, Milan, Italy) and 5-fluorouracil (Fluorouracil-TEVA, Pharmachemie, Haarlem, Netherlands). Freshly prepared stock solutions were the followings: 100 times dilution of the original carboplatin solution with 10 mg/ml concentration and 5000 times dilution of the original 5-fluorouracil solution with 50 mg/ml concentration. The original doxorubicin powder was also freshly dissolved first in sterile distilled water to get solution with 2 mg/ml concentration then was diluted 5000 times. Stock

solutions were dissolved in sterile McCoy's tissue medium. The chromatography-purified sodium salt of 4-thiouridylate (designated as UD29), was prepared as we described earlier [19].

Study design

At first the body weight, blood glucose and plasma insulin levels were determined in 10 control and 10 obese *db/db* mice to check for the development of insulin resistance and DMT2. Changes in peripheral blood cell counts and bone marrow cellularity, the total nucleated cell number of the femoral bone marrow, were also investigated. Thereafter, the myelopoiesis of these two groups were compared by studying the common progenitors of the phagocytic cells, the granulocyte-macrophage colony forming units (CFU-GM). Frequency of CFU-GM progenitors was estimated in a specific soft gel colony assay and total femoral CFU-GM content was calculated from the cellularity and the frequency of CFU-GM progenitor cells.

In the next round of experiments the myelopoiesis was 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 blood cell counts, femoral cellularity, frequency of CFU-GM progenitor cells and total femoral CFU-GM content were determined 48 hours following the injection of carboplatin.

To study the direct effects of cytotoxic drugs on CFU-GM progenitor cells, nucleated bone marrow cells collected from control and *db/db* mice were cultured *in vitro* in the presence of increasing concentrations (0-0.001-10 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. In addition to these widely used cytotoxic drugs, the in vitro effects of increasing concentrations (1-300 μM) of 4-thiouridylate, a chemically modified mononucleotide, on CFU-GM progenitor cells were also investigated in the same way.

Measurement of blood glucose and plasma insulin levels

Blood samples were taken from retroorbital plexus of the mice after overnight fasting in the morning. Blood glucose concentrations were determined directly from one drop of blood samples using AccuCheck (Roche Diagnostics, Mannheim, Germany). For insulin determination the blood samples were centrifuged (Centrifuge 5415R; Eppendorf AG, Hamburg, Germany) for 2 minutes at 4°C and 10,000g; then, the plasma was aliquoted, frozen, and stored at -70°C. After thawing plasma samples were used directly without any extraction processes. Plasma insulin levels were determined by means of a commercially available radioimmunoassay kit (RK-400CT, Institute of Isotopes of the Hungarian Academy of Sciences, Budapest, Hungary).

Bone marrow samples

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. The concentration of nucleated bone marrow cells in this suspension was determined using a hemocytometer and the cellularity of the bone marrow was calculated as the product of the volume of the suspension and the cell concentration.

CFU-GM assay

Special soft-gel cultures were prepared as described earlier [20]. An inoculate of 2×10^5 /ml bone marrow cells were plated in petri dishes (Greiner, Nürtingen, Germany) and were grown in McCoy's 5A modified medium (Sigma-Aldrich, Budapest, Hungary) supplemented with amino acids, Na pyruvate, NaHCO_3 , antibiotics (streptomycin, penicillin) and 20% foetal bovine serum. Conditioned medium of WEHI-3B cells was also added as a source of colony stimulating factors. Methylcellulose (Methocel, 3000-5000 centipoises; FLUKA, Buchs, Switzerland) at 1.2% was used as the support matrix for semi-solid cultures. Cultures were grown in triplicates for 7 days at 37 °C at 100% relative humidity in an atmosphere containing 5% CO_2 . Colonies were counted under stereomicroscope (Olympus, Hamburg, Germany). At the end of the incubation period colonies, defined as cell groups consisting of at least 50 cells, were counted under a stereomicroscope (Olympus, Hamburg, Germany).

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 $p < 0.05$.

Results

Characteristics of the untreated obese *db/db* mice

The *db/db* mice exhibit obesity, hyperglycemia, insulin resistance, and other phenotypes, which are physiologically relevant to DMT2. Control mice were heterozygous, expressing leptin receptors, had normal glucose metabolism and weight. They were used as a non-diabetic healthy control (Fig. 1a, b).

Bone marrow function was evaluated by total cellularity, frequency of granulocyte-macrophage progenitors (CFU-GM) 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 (Fig. 1c).

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 10^5 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. Colony forming capability of CFU-GM progenitors of obese *db/db* mice was not impaired in the absence of cytotoxic drugs. According to this, hemopoiesis seemed to be intact in these obese, diabetic animals (Fig. 1c).

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

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 both in control and *db/db* obese mice. Carboplatin significantly decreased the cellularity (Fig.

2a) and the frequency of CFU-GM progenitors (Fig. 2b) in groups of *db/db* mice compared to non-obese mice. As a consequence the total CFU-GM pool became significantly lower (Fig. 2c) 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.

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 (Fig. 3a). This decrease was primarily due to decreased number of circulating neutrophil granulocytes. Absolute neutrophil counts dropped into the very low range. In carboplatin-treated control mice the absolute neutrophil count was twice as high as in carboplatin-treated *db/db* mice ($1.35 \pm 0.09 \times 10^9/L$) while there were not seen a significant decrease in control mice after the carboplatin administration on the second day (Fig. 3b).

In vitro sensitivity of granulocyte-macrophage progenitor cells (CFU-GM) of control and obese *db/db* mice to common cytotoxic drugs

To evaluate whether an increased drug exposure resulted in the increased *in vivo* myelotoxicity of carboplatin, we used the same carboplatin concentrations *in vitro* in cultures of CFU-GM cells isolated from healthy non-obese controls and mice with obesity-associated diabetes mellitus. In the presence of increasing concentrations of carboplatin a dose dependent decrease in CFU-GM numbers were observed in bone marrow cell cultures of both control and *db/db* mice. However, the sensitivity of CFU-GM cells of obese *db/db* mice was increased to carboplatin, dose-response curve of cells from *db/db* mice was shifted to lower concentrations comparing with that of control mice (Fig 4a).

To know whether the increased toxicity is specific for carboplatin on the CFU-GM progenitor cells of *db/db* mice, other well-known anticancer drugs were tested. This is shown on Figure 4 that colony numbers (representing CFU-GMs) were significantly lower also for doxorubicin (Fig. 4b) and 5-fluorouracil (Fig. 4c), respectively - at any concentrations in cell cultures of obese *db/db* mice than in controls. These drugs were similarly more toxic to CFU-GM progenitors of *db/db* than control mice.

Toxicity of 4-thiouridylate, an anticancer drug under investigation, on granulocyte-macrophage progenitor cells (CFU-GM) of control and obese *db/db* mice

These findings highlighted that to find new anticancer agents with less toxicity especially on progenitor cells of mice (perhaps human patients) with obesity-associated diabetes mellitus has a great importance. We chose 4-thiouridylate, a chemically modified mononucleotide to study its toxicity on CFU-GM progenitor cells of *db/db* mice. In our previous work we found that it had a promising antileukemic effect *in vivo* on acute lymphoid leukemia human cell lines transplanted them into mice with serious combined immune deficiency (SCID). Therapeutic index was good based on the 14-fold less toxicity of 4-thiouridylate on healthy human CFU-GM progenitors than JY leukemic cell line [21]. Four-thiouridylate, analogously to the previously tested 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 4-thiouridylate (Fig. 5). However 4-thiouridylate, an anticancer drug under investigation, was by far the least toxic for CFU-GM of *db/db* mice. In addition, the differences were higher in diabetic *db/db* mice. Doxorubicin was the most, and carboplatin was the least toxic to the CFU-GM progenitor cells among the studied common

cytotoxic drugs. The fifty percent inhibitory concentration of 4-thiouridylate 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 (Table 1). This may be an advantage in therapy of malignant diseases for diabetic and obese patients, if 4-thiouridylate proved to be a proper drug for clinical use in the future.

Discussion

Although there is no complete agreement about the quantitative changes of multipotent progenitor cells in bone marrow of diabetic mice, similarly to our results (Fig 1c) other investigators could not observe decreased number of lineage-committed progenitor cells in bone marrow of diabetic mice [22] and even expansion of myeloid progenitors was described [23]. At the same time several data suggest a negative impact of diabetes on stem cell mobilization from bone marrow probably due to alteration of niche function and not to a direct damage of progenitor cells [23-24].

It should be noted that the above mentioned results were described in diabetes mellitus type 1 both in mice and patients. However hyperglycaemia is a characteristics of both DM type 1 and type 2, insulin resistance and obesity is associated only with DMT2, resulting in more complex disturbances in *db/db* mice. *Db/db* mouse is an accepted competent model of human obesity and obesity-associated diabetes, because of a spontaneous autosomal recessive mutation on chromosome 4 that inhibits the expression of leptin receptors. The developing leptin resistance causes obesity and DMT2.

The observed increased myelotoxicity of carboplatin in *db/db* mice also supports the idea that alteration of the function rather than the number of progenitor cells may better reflect

the deleterious effects of obesity-associated diabetes (Fig 2, Fig 4). Altered bone marrow function in DM is also suggested by the decreased number and function of bone marrow derived angiogenic progenitors which thought to contribute to the impaired endothelial renewal [25]. Additionally, it has been reported recently that transplantation of bone marrow derived mesenchymal stem cells from non-diabetic animals improved delayed wound healing in a diabetic rat model [26] which, although only indirectly, also suggests a potential dysfunction of bone marrow progenitor cells in DM. A similar observation, showing decreased in vitro proliferative capacity and decreased in vivo direct engraftment potential of bone marrow-derived mesenchymal stem cells originated from obese and diabetic *db/db* mice, provided a more direct proof that stem cell impairment is a significant complication of DMT2 in mice [27].

As *db/db* mice are both obese and diabetic it is possible that changes in pharmacokinetics in obesity may also play a role in the observed increased in vivo myelotoxicity of carboplatin, by resulting in higher drug exposure [4]. Total drug exposure is primarily, but not only, determined by the dose and dose schedule and it is well known that drug exposure is critical in determination of both beneficial and toxic effects of cancer chemotherapy. Large studies suggest that the beneficial impact of adjuvant chemotherapy is diminished when full doses of therapy are not given [28-31]. Based on these studies, it is generally recommended that chemotherapeutical doses should not be reduced below 85% of the standard doses over the entire course of the treatment.

However contradictory data are available regarding the association of increased toxicity of chemotherapy in obese or diabetic individuals, some observations reported increased therapy-related myelotoxicity in obese patients, e.g. in obese children with acute myeloid leukaemia (AML) [32]. Meloni et al. [33] found delayed granulocyte recovery and

increased incidence of infections in obese adults with *de novo* AML receiving high dose chemotherapy and autologous stem cell transplantation.

Although DM seems to be a strong independent factor influencing mortality of patients with malignancies [34-35], there are only a few observations about an increased myelotoxicity during chemotherapy. Srokowsky et al. [36] demonstrated in elderly breast cancer patients that diabetic patients were more likely to experience toxicities of chemotherapy than were non-diabetic patients. Diabetic patients were more likely to be hospitalized for any cause, for toxicity in general, and for infection or fever, neutropenia and anaemia. Majority of these patients received doxorubicin-based chemotherapy schedules. Our data might suggest functional damages of bone marrow progenitor cells in diabetic patients as we found that CFU-GM progenitors from mice with obesity-associated diabetes were more sensitive to doxorubicin (Fig 4b).

Our previous results were the first observations of increased toxicity of cytotoxic agents to granulocyte-macrophage progenitor cells of obese, insulin resistant Zucker rats *in vitro*. We introduced new methods for evaluating pathophysiological processes associated with obesity and insulin resistance. Although there was no difference between the obese and the lean control animals in number and proliferation of the haemopoietic progenitors in bone marrow, *in vitro* exposure to carboplatin, doxorubicin and 5-fluorouracil showed higher sensitivity of CFU-GM progenitors from Zucker obese rats to the toxicity of anticancer drugs than in bone marrow cells from control rats. According to our *in vitro* model the therapy-related myelotoxicity might be not only due to the different pharmacokinetic parameters or changes in drug exposure but also to the dysfunction of haemopoietic progenitor cells [15].

Our current *in vitro* results on CFU-GM from *db/db* mice correspond to our previous results and strongly suggest that increased vulnerability of progenitor cells may contribute to increased therapy-related myelotoxicity in obesity-associated diabetes (Fig 4). We tested the

sensitivity of the CFU-GM progenitor cells to three widely used cytotoxic drugs with different mechanism of action. Carboplatin forms reactive molecular species that alkylate nucleophilic groups on DNA bases. Doxorubicin intercalates between DNA base pairs, interacts with topoisomerase II and generates free radicals. Five-fluorouracil belongs to the antimetabolites. A common dose limiting side effect of all of these cytotoxic agents is myelosuppression similarly to other antiproliferative treatments,

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. As a consequence anemia, neutropenia and thrombocytopenia are developed. Neutropenia has the highest influence on survival by higher risk for serious infections, which are the leading cause of mortality during chemotherapy of cancer patients. A single intraperitoneal dose of carboplatin resulted in serious neutropenia in mice with obesity-associated diabetes as early as 2 days after administration while there were no any differences in absolute neutrophil counts in control mice (Fig 3).

According to our results, obesity-associated diabetes has direct significant effect on an important target cell population of cytotoxic drugs, namely CFU-GM, the common ancestors of granulocytes and macrophages with serious neutropenia. Toxicity of each tested anticancer drug with different mode of actions was similarly double to 5 times higher for CFU-GM progenitors from mice with obesity-associated diabetes than those of controls (Table 1). There

is only one other similar observation, *in vitro* 5-fluorouracil could damage progenitors of the *db/db* mice more than progenitors of the controls [37].

Our previous findings that insulin itself even insulin sensitizing had myeloprotective effect and defended CFU-GM progenitor cells against 5-fluorouracil-caused damage [16-18] supported that insulin resistance might be an opposite effect. We also demonstrated that CFU-GM progenitors of Goto-Kakizaki rats had higher sensitivity against carboplatin, and doxorubicin and 5-fluorouracil *in vitro* than CFU-GM cells of non-insulin resistant control rats [15]. Goto-Kakizaki rats are non-obese but insulin resistant animals thus our results suggest that insulin resistance may involve CFU-GM progenitor cells. Insulin and insulin-like growth factor are well-known early-acting growth factors in hemopoiesis, and they even share some insulin receptors, which have good affinity to both of them. If insulin resistance involves CFU-GM progenitors, both their survival and proliferation are affected and they become more sensitive to any agents with apoptotic and antiproliferative mode of actions.

Insulin resistance, which characterizes both obesity-associated diabetes and obesity may alter tumour cells, too. In majority of cases it does not mean that tumour cells themselves become insulin resistant. On the contrary, Novosyadlyy et al [38] found highest density of insulin receptors on breast tumor cells in insulin resistant but nonobese diabetic animals with accelerated tumor progression. Insulin receptors have a great role in autocrine loops of some cancer cells with accelerated tumor progression and their increased numbers explain their increased sensitivity to hyperinsulinemia associated with diabetes mellitus or insulin resistance with or without obesity. Insulin receptor abundance and its phosphorylation is characteristic for breast cancer cells and are positively related to poor survival [39]. Enhanced proliferation rate and survival of tumour cells are well-known result of activated signaling through Akt pathway which propagates not only the metabolic but also growth factor like effects of insulin [40]. *De novo* activation of the autocrine loop through insulin receptor

signalling, also represent a mechanism of resistance to any antiproliferative or apoptosis inducer anticancer drugs.

On the other hand high insulin receptor density does not accelerate tumour growth if there is lack of phosphorylation, if e.g. breast cancer cells express high levels of ectonucleotide pyrophosphatase/phosphodiesterase which lead to insulin resistance of tumour cells [40]. Therapy resistance may develop against the new insulin receptor tyrosine kinase inhibitors such as linsitinib.

Summarizing the above mentioned data, in obesity-associated diabetes therapeutic window of anticancer treatment might be narrower not only because of the higher myelotoxicity but also the higher resistance of tumor cells. This highlighted importance of new anticancer agents with less toxicity especially to progenitor cells of diabetic patients. As a first step we tested toxicity of 4-thiouridylate, an anticancer drug under investigation, on granulocyte-macrophage progenitor cells (CFU-GM) of control and obese *db/db* mice (Fig 5). A mononucleotide, 4-thiouridylate showed anti-proliferative activity on various leukemic and solid tumour cell lines [19, 41]. 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 including receptors, involved in the regulation of cell cycle or cell death [41]. 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. Four-thiouridylate was by far the least toxic for CFU-GM of *db/db* mice, in addition differences were higher for CFU-GM progenitors of mice with obesity-associated diabetes than those of control animals (Table 1). Lower toxicity may be based on its mode of action, as 5-thiouridylate does not penetrate cell membrane and cause direct damage in DNA, it results in probably a receptor-activated apoptosis.

According to our current observations, proliferation and survival of CFU-GM progenitors were also affected in *db/db* mice but this could be detected only through their higher vulnerability to cytotoxic drugs. The most significant aspect of our new results is to emphasize dysfunction of haemopoietic progenitor cells in obesity-associated diabetes. This dysfunction could not be detected under physiologic circumstances. However, the vulnerability of CFU-GMs 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. The early occurrence of severe neutropenia – as early as 48 hours after administration of carboplatin – also suggests direct damage to not only progenitor cells but also circulating mature cells (Fig 3). Disorders in cell metabolism and receptorial functions may be responsible for the increased vulnerability of progenitor cells 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-associated diabetes and might improve the outcome of chemotherapy of malignant diseases.

Acknowledgments This work was supported by the common fund of European Union and Hungarian government (TÁMOP-4.1.1.F-13/1-2013-0004).

Conflict of interest All authors declare that there is no conflict of interest.

References

1. Rodriguez C, Patel AV, Calle EE, Jacobs EJ, Chao A, Thun MJ (2001) Body mass index, height, and prostate cancer mortality in two large cohorts of adult men in the United States. *Cancer Epidemiol Biomarkers Prev* 10:345-353
2. Blouin RA, Warren GW (1999) Pharmacokinetic considerations in obesity. *J Pharm Sci* 88:1-7 Review
3. Griggs JJ, Mangu PB, Anderson H, Balaban EP, Dignam JJ, Hryniuk WM, Morrison VA, Pini TM, Runowicz CD, Rosner GL et al. (2012) Appropriate chemotherapy dosing for obese adult patients with cancer: American Society of Clinical Oncology clinical practice guideline. American Society of Clinical Oncology. *J Clin Oncol* 30:1553-1561 Review
4. De Jonge ME, Mathot RA, Van Dam SM, Beijnen JH, Rodenhuis S (2002) Extremely high exposures in an obese patient receiving high-dose cyclophosphamide, thiotepa and carboplatin. *Cancer Chemother Pharmacol* 50:251-255
5. Ekhardt C, Rodenhuis S, Schellens JH, Beijnen JH, Huitema AD (2009) Carboplatin dosing in overweight and obese patients with normal renal function, does weight matter? *Cancer Chemother Pharmacol* 64:115-122

6. Miyahara T, Mochinaga S, Kimura S, Aragane N, Yakabe T, Morita S, Okudaira K, Fujito H (2013) Effects of tumor type, degree of obesity, and chemotherapy regimen on chemotherapy dose intensity in obese cancer patients. *Cancer Chemother Pharmacol* 71:175-182.
7. Thompson P, Wheeler HE, Delaney SM, Lorier R, Broeckel U, Devidas M, Reaman GH, Scorsone K, Sung L, Dolan ME, Berg SL (2014) Pharmacokinetics and pharmacogenomics of daunorubicin in children: a report from the Children's Oncology Group *Cancer Chemother Pharmacol* 74:831-838
8. Sparano JA, Wang M, Zhao F, Stearns V, Martino S, Ligibel JA, Perez EA, Saphner T, Wolff AC, Sledge GW Jr, Wood WC, Fetting J, Davidson NE (2012) Obesity at diagnosis is associated with inferior outcomes in hormone receptor-positive operable breast cancer. *Cancer* 118:5937-5946
9. Schmitt A, Gladiëff L, Lansiaux A, Bobin-Dubigeon C, Etienne-Grimaldi MC, Boisdron-Celle M, Serre-Debauvais F, Pinguet F, Floquet A, Billaud E, Le Guellec C, Penel N, Campone M, Largillier R, Capitain O, Fabbro M, Houede N, Medioni J, Bougnoux P, Lochon I, Chatelut E (2009) A Universal Formula Based on Cystatin C to Perform Individual Dosing of Carboplatin in Normal Weight, Underweight and Obese Patients. *Clin Cancer Res* 15:3633-3639
10. Brown DL, Masselink AJ, Lalla CD (2013) Functional range of creatinine clearance for renal drug dosing: a practical solution to the controversy of which weight to use in the Cockcroft-Gault equation. *Ann Pharmacother* 47:1039-1044
11. Wright JD, Tian C, Mutch DG, Herzog TJ, Nagao S, Fujiwara K, Powell MA (2008) Carboplatin dosing in obese women with ovarian cancer: a Gynecologic Oncology Group study. *Gynecol Oncol* 109:353-358

12. Chen S, Chen CM, Zhou Y, Zhou RJ, Yu KD, Shao ZM (2012) Obesity or overweight is associated with worse pathological response to neoadjuvant chemotherapy among Chinese women with breast cancer. *PLoS One* 7:e41380, doi: 10.1371
13. Au-Yeung G, Webb PM, DeFazio A, Fereday S, Bressl M, Mileskin L (2014) Impact of obesity on chemotherapy dosing for women with advanced stage serous ovarian cancer in the Australian Ovarian Cancer Study (AOCS). *Gynecol Oncol* 133:16-22
14. Kashiwabara K, Yamane H, Tanaka H (2013) Toxicity and prognosis in overweight and obese women with lung cancer receiving carboplatin-paclitaxel doublet chemotherapy. *Cancer Invest* 31:251-257
15. Géresi K, Benkő K, Szabó B, Megyeri A, Peitl B, Szilvássy Z, Benkő I (2012) 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* 696:172-178
16. Benkő I, Djazayeri K, Abrahám C, Zsuga J, Szilvássy Z (2003) Rosiglitazone-induced protection against myelotoxicity produced by 5-fluorouracil. *Eur J Pharmacol* 477:179-182
17. Djazayeri K, Szilvássy Z, Peitl B, Németh J, Nagy L, Kiss A, Szabó B, Benkő I (2005) Accelerated recovery of 5-fluorouracil-damaged bone marrow after rosiglitazone treatment. *Eur J Pharmacol* 522:122-129
18. Djazayeri K, Szilvássy Z, Benkő K, Rózsa B, Szabó B, Szentmiklósi AJ, Benkő I (2006) Effect of rosiglitazone, an insulin sensitizer, on myelotoxicity caused by repeated doses of 5-fluorouracil. *Pharmacol Res* 53:156-161
19. Kemény-Beke A, Berényi E, Facskó A, Damjanovich J, Horváth A, Bodnár A, Berta A, Aradi J (2006) Antiproliferative effect of 4-thiouridylate on OCM-1 uveal melanoma cells. *Eur J Ophthalmol* 16:680-685

20. Benkő I, Hernádi F, Megyeri A, Kiss A, Somogyi G, Tegyei Z, Kraicsovits F, Kovács P (1999) Comparison of the toxicity of fluconazole and other azole antifungal drugs to murine and human granulocyte-macrophage progenitor cells *in vitro*. *J Antimicrob Chemother* 43:675-681
21. Berényi E, Benkő I, Vámosi G, Géresi K, Tárkányi I, Szegedi I, Lukács L, Juhász I, Kiss C, Fésüs L, Aradi J (2011) In vitro and in vivo activity of 4-thio-uridylate against JY cells, a model for human acute lymphoid leukemia. *Biochem Biophys Res Commun* 410:682-687
22. Oikawa A, Siragusa M, Quaini F, Mangialardi G, Katare RG, Caporali A, van Buul JD, van Alphen FP, Graiani G, Spinetti G, Kraenkel N, Prezioso L, Emanuelli C, Madeddu P (2010) Diabetes mellitus induces bone marrow microangiopathy. *Arterioscler Thromb Vasc Biol* 30:498-508
23. Ferraro F, Lymperi S, Méndez-Ferrer S, Saez B, Spencer JA, Yeap BY, Masselli E, Graiani G, Prezioso L, Rizzini EL, Mangoni M, Rizzoli V, Sykes SM, Lin CP, Frenette PS, Quaini F, Scadden DT (2011) Diabetes impairs hematopoietic stem cell mobilization by altering niche function. *Sci Transl Med* 3:104ra101. doi: 10.1126/scitranslmed.3002191
24. DiPersio JF (2011) Diabetic stem-cell "mobilopathy". *N Engl J Med* 365:2536-2538
25. Gilbert RE (2013) Augmenting endothelial repair in diabetes: role of bone marrow-derived cells. *Can J Diabetes* 37:315-318
26. Wan J, Xia L, Liang W, Liu Y, Cai Q (2013) Transplantation of bone marrow-derived mesenchymal stem cells promotes delayed wound healing in diabetic rats. *J Diabetes Res* 2013:647107. doi: 10.1155/2013/647107
27. Shin L, Peterson DA (2012) Impaired therapeutic capacity of autologous stem cells in a model of type 2 diabetes. *Stem Cells Transl Med* 1:125-135

28. Budman DR, Berry DA, Cirincione CT, Henderson IC, Wood WC, Weiss RB, Ferree CR, Muss HB, Green MR, Norton L, Frei E 3rd (1998) Dose and dose intensity as determinants of outcome in the adjuvant treatment of breast cancer. The Cancer and Leukemia Group B. *J Natl Cancer Inst* 90:1205-1211
29. Colleoni M, Price K, Castiglione-Gertsch M, Goldhirsch A, Coates A, Lindtner J, Collins J, Gelber RD, Thürlimann B, Rudenstam CM (1998) Dose-response effect of adjuvant cyclophosphamide, methotrexate, 5-fluorouracil (CMF) in node-positive breast cancer. International Breast Cancer Study Group. *Eur J Cancer* 34:1693-1700
30. Morrow T, Siegel M, Boone S, Lawless G, Carter W (2002) Chemotherapy dose intensity determination as a quality of care measure for managed care organizations in the treatment of early-stage breast cancer. *Am J Med Qual* 17:218-224
31. Griggs JJ, Sorbero ME, Lyman GH (2005) Undertreatment of obese women receiving breast cancer chemotherapy. *Arch Intern Med* 165:1267-1273
32. Canal P, Chatelut E, Guichard S (1998) Practical treatment guide for dose individualisation in cancer chemotherapy. *Drugs* 56:1019-1038 Review
33. Meyerhardt JA, Tepper JE, Niedzwiecki D, Hollis DR, McCollum AD, Brady D, O'Connell MJ, Mayer RJ, Cummings B, Willett C, Macdonald JS, Benson AB 3rd, Fuchs CS (2004) Impact of body mass index on outcomes and treatment-related toxicity in patients with stage II and III rectal cancer: findings from Intergroup Trial 0114. *J Clin Oncol* 22:648-657
34. Satariano WA, Ragland DR (1994) The effect of comorbidity on 3-year survival of women with primary breast cancer. *Ann Intern Med* 120:104-110

35. Yancik R, Ganz PA, Varricchio CG, Conley B (2001) Perspectives on comorbidity and cancer in older patients: approaches to expand the knowledge base. *J Clin Oncol* 19:1147-1151
36. Srokowski TP, Fang S, Hortobagyi GN, Giordano SH (2009) Impact of diabetes mellitus on complications and outcomes of adjuvant chemotherapy in older patients with breast cancer. *J Clin Oncol* 27:2170-2176
37. Umemoto Y, Tsuji K, Yang FC, Ebihara Y, Kaneko A, Furukawa S, Nakahata T (1997) Leptin stimulates the proliferation of murine myelocytic and primitive hematopoietic progenitor cells. *Blood* 90:3438-3443
38. Novosyadlyy R, Lann DE, Vijayakumar A, Rowzee A, Lazzario DA, Fierz Y, Carboni JM, Gottardis MM, Pennisi PA, Molino AA, Kurshan N, Mejia W, Santopietro S, Yakar S, Wood TL, LeRoith D (2010) Insulin-mediated acceleration of breast cancer development and progression in a nonobese model of type 2 diabetes. *Cancer Res* 70:741-751
39. Law JH, Habibi G, Hu K, Masoudi H, Wang MY, Stratford AL, Park E, Gee JM, Finlay P, Jones HE, Nicholson RI, Carboni J, Gottardis M, Pollak M, Dunn SE (2008) Phosphorylated insulin-like growth factor-I/insulin receptor is present in all breast cancer subtypes and is related to poor survival. *Cancer Res* 68:10238-10246
40. Belfiore A, Malaguarnera R (2011) Insulin receptor and cancer. *Endocr Relat Cancer* 18:R125-147
41. Beck Z, Kis A, Berényi E, Kovács P, Fésüs L, Aradi J (2009) 4-Thio-uridylylate (UD29) interferes with the function of protein -SH and inhibits HIV replication in vitro. *Pharmacol Rep* 61:343-347

Legends to Figures and Table

Figure 1 Characteristics of obese *db/db* mice in comparison with *db/-* control mice. Body weight (a), blood glucose and plasma insulin level (b), total cellularity of bone marrow and frequency of granulocyte-macrophage progenitor cells (CFU-GM) (c) in twelve-week-old control, and obese, diabetic *db/db* mice. (n=10 in each group, values are means \pm S.E.M., ***P<0.001 compared with control, non-obese, non-diabetic mice).

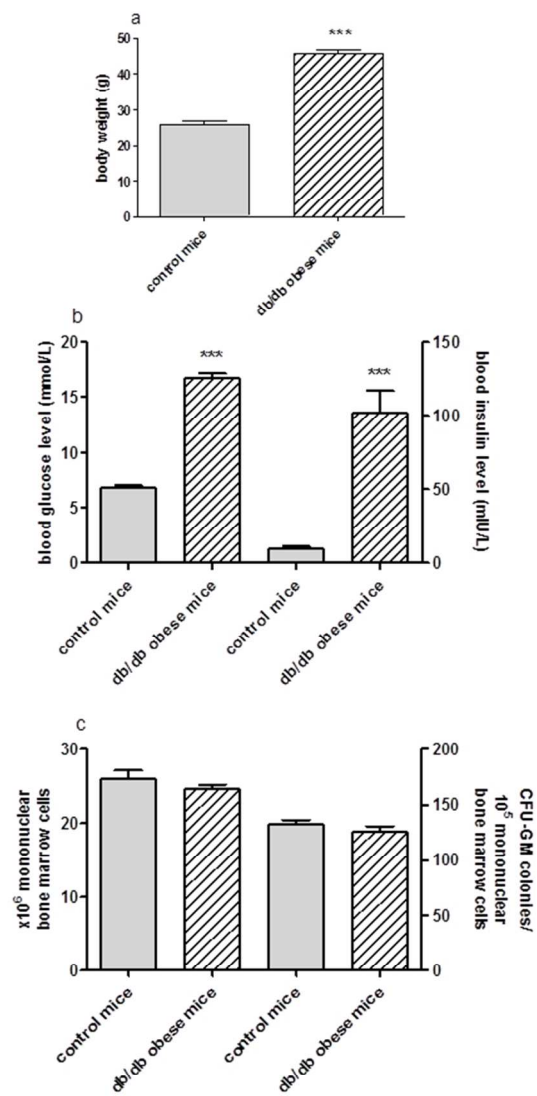
Figure 2 Effect of *in vivo* administration of carboplatin on cellularity (a), CFU-GM colony numbers (b) and total CFU-GM content (c) of femoral bone marrow in control and obese, diabetic *db/db* mice after 48 hours of a single 100 mg/kg dose. (n=10 in each group, values are means \pm S.E.M., *P<0.05, ***P<0.001 compared with control mice).

Figure 3 White blood cell (a) and absolute neutrophil (b) counts in vehicle-treated control mice and after 48 hours of a single 100 mg/kg dose of carboplatin (CP) in 12-week-old control, and obese, diabetic *db/db* mice. (n=10 in each group, values are means \pm S.E.M., **P<0.01 and ***P<0.001 compared with carboplatin treated control mice).

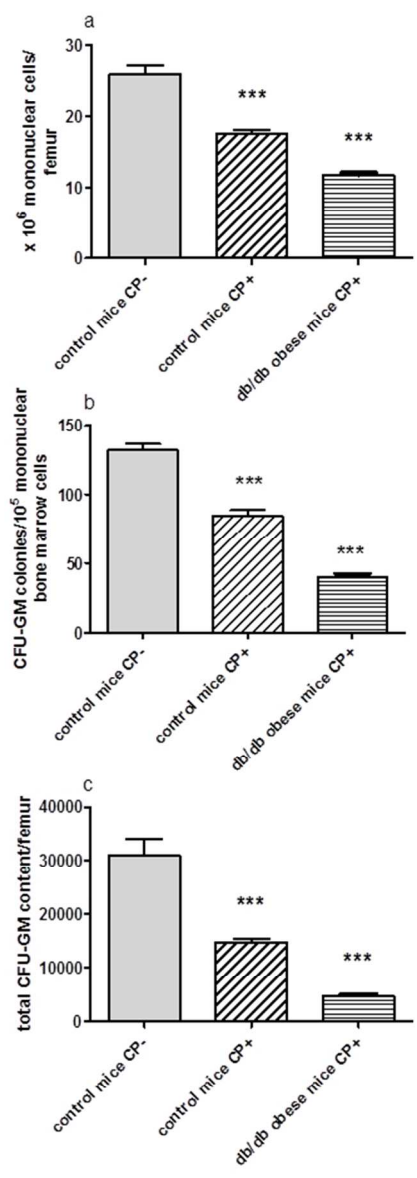
Figure 4 Effect of carboplatin (a), doxorubicin (b) and 5-fluorouracil (c) on CFU-GM colony numbers in control and obese, diabetic *db/db* mice in special soft-gel cultures. Cultures were grown in the presence of increasing concentrations of carboplatin, doxorubicin and 5-fluorouracil. (n=10 in each group, values are means \pm SEM, **P<0.01, ***P<0.001 compared with control mice).

Figure 5. Effect of 4-thiouridylate on CFU-GM colony numbers in control and obese, diabetic *db/db* mice in special soft-gel cultures. Cultures were grown in the presence of increasing concentrations of 4-thiouridylate (n=10 in each group, values are means \pm SEM, *P<0.05, **P<0.01, compared with control mice).

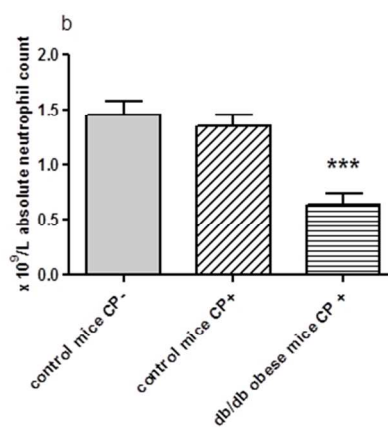
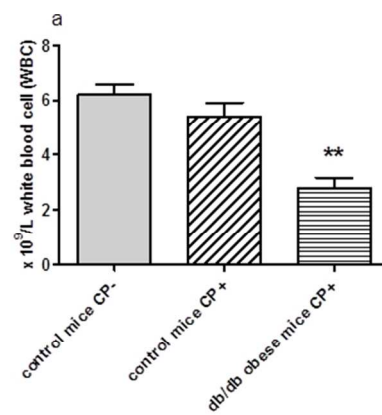
Table 1. Fifty percent inhibitory concentrations (IC50) of carboplatin, doxorubicin, 5-fluorouracil and 4-thiouridylate on CFU-GM cells from control *db/-* and obese, *db/db* mice.



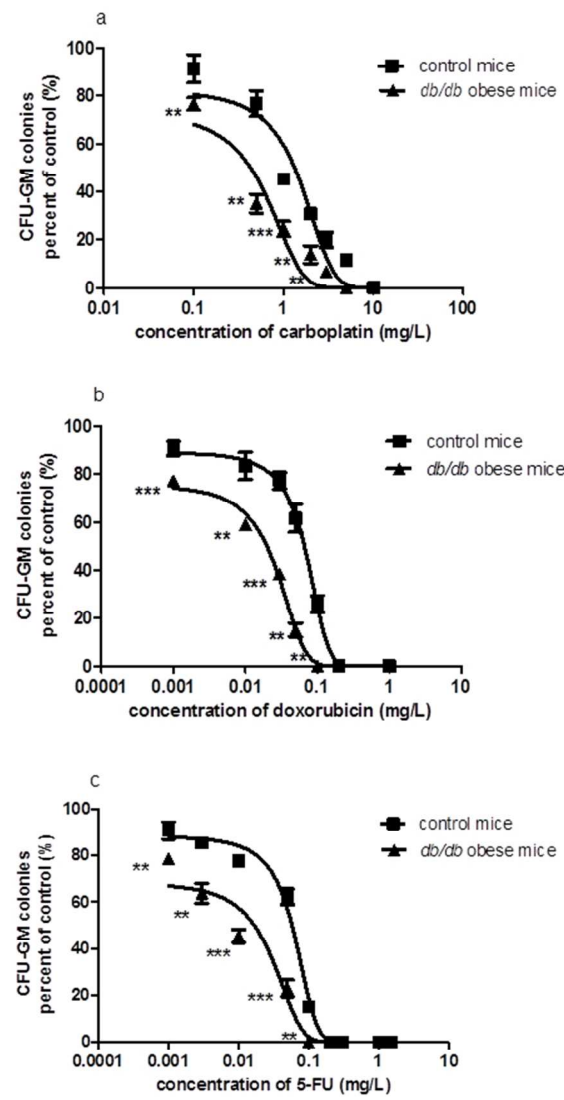
190x254mm (96 x 96 DPI)



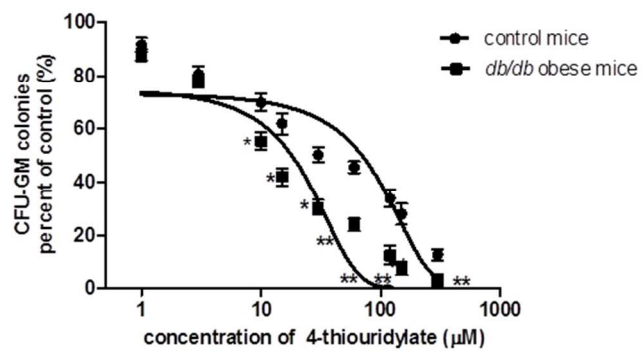
190x254mm (96 x 96 DPI)



190x254mm (96 x 96 DPI)



190x254mm (96 x 96 DPI)



190x254mm (96 x 96 DPI)

	IC50 (μ M)	IC50 (μ M)
	control mice	<i>db/db</i> obese mice
carboplatin	2.22	0.46
doxorubicin	0.0082	0.0018
5-fluorouracil	0.03	0.015
4-thiouridylate	33.82	11.73

190x254mm (96 x 96 DPI)