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Myeloprotective effects of rosiglitazone, an insulin sensitizer, on 5-fluorouracil-induced toxicity

Thesis for the Degree of Doctor of Philosophy (Ph.D.)

By

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List of Abbreviations

ANC :	Absolute neutrophil count
ATRA :	All trans retinoic acid
BFU-E :	Burst-forming units
BM :	Bone marrow
BMC :	Bone marrow cell count
CFU-GM :	Granulocyte-macrophage colony-forming units (Granulocyte–macrophage progenitor cells)
CSF :	Colony stimulating factors
5-FU :	5-fluorouracil
G-CSF :	Granulocyte colony stimulating factors
GF :	Growth factor
GIR :	Glucose infusion rate
HDL :	high density lipoprotein
HSC :	Hematopoietic stem cells
HEGC :	Hyperinsulinemic euglycemic glucose clamp
IGF :	Insulin like growth factor
IL :	Interleukins
LDL :	Low density lipoprotein
M-CSF :	Macrophage colony-stimulating factor
NIDDM :	Non Insulin Dependent Diabetes Mellitus
PPARγ :	Peroxisome-proliferator-activated receptor-gamma
PPAR :	Peroxisome-proliferator-activated receptor
RIA:	Radioimmunoassay
SCF :	Stem cell factor
TZD :	Thiazolidinediones
WBC :	White blood cell count
WHO :	World Health Organization

1. INTRODUCTION

In the year 2000, malignant tumors were responsible for 12 per cent of the nearly 56 million deaths worldwide from all causes. In many countries, more than a quarter of deaths are attributable to cancer. In 2000, 5.3 million men and 4.7 million women developed malignant tumors and altogether 6.2 million died from the disease. The report also reveals that cancer has emerged as a major public health problem in developing countries, matching its effect in industrialized nations. Mortality has always been high among patients with malignant diseases, but even today despite all the complex anticancer therapy the mortality rate stands at about 50% within 5 years in cases required systemic treatment. As a result the 2nd leading cause of death following the cardiovascular diseases has become malignancy (WHO report 2003).

1.1. Myelotoxicity induced by cytostatic agents

Myelosuppression is the most common dose-limiting side effect of chemotherapy during the complex anticancer treatment and together with its complications can also be the most lethal. All hematopoietic cells divide rapidly, regardless of their developmental stage, and are therefore vulnerable to chemotherapy. Proliferating progenitor cells that produce the mature granulocytes, erythrocytes, and thrombocytes in the peripheral circulation are commonly destroyed. As immature cells in the marrow and preexisting mature cells are destroyed, the nadir becomes apparent, usually 7-14 days after chemotherapy. At the same time, cells in the bone marrow are maturing and are ready to be released into the peripheral blood. Within a short period of time (3-4 weeks), the nadir will resolve, if the regeneration is not disturbed by the repeated doses of the cytostatic agents. Thus the treatment schedules have to be suspended until the severe neutropenia improves, and tumour may be further growing and malignant cells may even overflow the body in the meantime. However, when high doses are administered, the stem cell population may fail to repopulate quickly enough, resulting in a

prolonged nadir period. Chemotherapy-induced myelosuppression is the most common dose-limiting side effect of cancer chemotherapy. Antineoplastic therapy-associated hematopoietic toxicity (myelosuppression) will often result in neutropenia, anemia and thrombocytopenia . During the period of myelosuppression, patients may be at an increased risk of infection or bleeding or may experience symptoms from anemia.

The majority of chemotherapy drugs cause some degree of myelosuppression (Ozkan et al., 2005). Agents most active against cells that are cycling or those active during a specific phase of the cell cycle can produce rapid cytopenia. Because alkylating agents and nitrosoureas affect both cycling cells and noncycling cells, these drugs are more likely to destroy the marrow stem cells. Antimetabolites, vinca alkaloids, and antitumor antibiotics are most damaging to cells that are in a specific phase of the cell cycle; thus, myelosuppression is less severe with these agents. However, dose intensification and drug combinations can produce severe and prolonged neutropenia. (Dale, 2002).

Myelosuppression can be the dose-limiting toxicity, even in the case of newer agents such as paclitaxel, docetaxel, vinorelbine, and gemcitabine. Paclitaxel can cause neutropenia, with the severity dependent on the administration schedule, dose, extent of previous treatment, and pharmacological exposure to the drug. Primary or metastatic growths together with chemotherapy treatments will eventually result in myelosuppression (Ozkan et al., 2005), defined as a condition in which the bone marrow has a decreased production or even inhibited stem and progenitor cell proliferation and/or may take a prolonged period of time to return to "normal levels". Myelosuppression is characterized by the decrease in bone marrow (BM) cellularity, frequency and content of stem and progenitor cells. Granulocyte–macrophage progenitors (CFU-GM) are the most important suppressed group among hematopoietic cells. Suppression and underproduction of this group of progenitor cells will result in neutropenia which in turn exposes the patient to opportunistic infections and as neutrophil leukocytes have the shortest half-life in circulation their continuous renewal is essential to prevent the neutropenia induced by chemotherapeutic agent. The clinical sequel of neutropenia is manifested as infections, which are specific to patients with immunodeficiency not normally occuring in patients without this condition. Such infection in an immunosuppressed patient is considered to be serious and can cause high mortality rate. Such opportunistic infections

manifest symptoms late in the course of the disease resulting in delayed and ineffective treatment.

1.2. Neutropenia and associated opportunistic infections

Neutropenia specifically is a serious risk with chemotherapy, associated with increased opportunistic infections, complicated with use of intravenous antibiotics, hospitalization, and even death. The occurrence of febrile neutropenia can lead to dose reductions and delay in subsequent cycles of chemotherapy that may have a detrimental affect on overall survival and disease-free survival. (Wolf, et al., 2004). Also it is used as a marker of the degree of myelosuppression in patients undergoing chemotherapy.

The risks of infection and their complications are related to both the severity and duration of neutropenia (Kuhn, 2002). As mentioned before neutropenia remains the major reason for hospitalization during or after chemotherapy and is defined as a decrease in circulating neutrophils in the peripheral blood. The absolute neutrophil count (ANC) defines neutropenia. ANC is found by multiplying the percentage of bands and neutrophils on a differential by the total white blood cell count. An abnormal ANC is fewer than 1500 cells / mm3.

The severity of neutropenia is categorized as mild with an ANC of 1000-1500 cells / mm3, moderate with an ANC of 500-1000 cells /mm3, and severe with an ANC of fewer than 500 cells / mm3. The risk of bacterial infections related to the severity and duration of neutropenia. Patients are difficult to evaluate due to their decreased immune responses. The 48% to 60% of neutropenic patients who become febrile have an evaluated infection. Febrile neutropenia (FN.) is defined as a rise in auxiliary temperature above 38.5^{o}C for a duration of more than one hour while having an absolute neutrophil count of less than 0.5x10^{9}/L. FN and its severity depends on the dose intensity of the chemotherapy regimen, the patient's prior history of either radiation therapy or use of cytotoxic treatment, and comorbidities. The occurrence of FN. often causes subsequent chemotherapy delays or dose reductions. It may also lengthen hospital stay, increase monitoring, diagnostic and treatment costs, and reduce

patient quality of life (Dale 2002). Only 50% of infected patients will have microbiological determination of an organism. Gram positive infections account for approximately 60% to 70% of microbiologically determined infections and the condition will not improve unless Absolute Neutrophil Count (ANC) is normalized signifying the recovery of granulopoiesis in the bone marrow. Changes of the (BM) cellularity, frequency and content of granulocyte–macrophage progenitors (CFU-GM) together with the absolute neutrophil count (ANC) point to the bone marrow regeneration.

The only response may be fever and at times this may not be present. It is estimated that 80% of the infections that occur arise from endogenous microbial flora of the gastrointestinal or respiratory tract. When the neutrophil count is less than 500 cells/mm3, approximately 20% or more of febrile episodes will have an associated bacteremia caused principally by aerobic Gram negative bacilli (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) and Gram-positive cocci (coagulase-negative staphylococci, streptococci species, and *Staphylococcus aureus*).

Mucous membranes are most commonly affected e.g. oral, perianal, genitalia. Skin is the second most common infection site, manifesting as ulcers, abscesses, rashes, and delays in wound healing. Signs of infection, including warmth and swelling, may be absent in prolonged severe neutropenia, sepsis may easily develop with life-threatening gastrointestinal and pulmonary infections. Since the primary function of neutrophils is phagocytosis, neutropenia eliminates one of the body's primary defenses against bacterial infections. Infections, due to invasion and overgrowth of pathogenic microbes, increase in frequency and severity as ANC decreases. In addition, risk for severe infections increases when the nadir persists for more than 7-10 days. Signs of an infection may not be apparent with the inhibition of phagocytic cells.

Fungal infections also play a huge role in immune compromised patients. Chemotherapy-induced damage to the alimentary canal and respiratory tract mucosa facilitates the entry of infecting organisms; therefore, pneumonia and sinusitis are commonly seen. Inflammation at

the sites most commonly infected, should be assessed, including the periodontium, pharynx, lower esophagus, lung, perineum, anus, skin, and venous access exit sites.

It is a clinical experience, that despite the modern antibiotic and antifungal therapy, these serious infections will not improve until the absolute neutrophil count in peripheral blood is normalized (Bodey et al. 1994). Often the patient will remain exposed to many fungal, viral and bacterial infections without the benefit from efficient antibiotic or antifungal drugs, since neither will be entirely curative. In such cases the more intensive courses of antibiotics with higher doses are called for and that is obviously directly associated with higher level of side effects linked to antibiotic use. Therefore the neutropenic period is the most dangerous time and infections associated with neutropenia are the leading causes of the deaths of malignant diseases.

Such severe myelotoxicity, calls for a fractionated regime which should be adopted with time interval for healthy cells recovery, and such interval times during therapy will determine patient's survival. So if we could moderate the myelotoxicity most importantly many more healthy cells are preserved and also the interval between following cytostatic doses could get shorter, which means there will be a less chance for regrowth of tumor cells (Dolan et al., 2005).

In shorter time better recovery would be achieved. Chemotherapy-induced hematopoietic toxicity is a multifactorial challenge that influences the treatment of oncology patients; therefore it is essential to introduce means to provide myeloprotective effects (Nichols et al.1994). However chemotherapy–induced neutropenia will not be as life-threatening if it could be effectively prevented and treated.

1.3. Role of growth factors in hematopoiesis

1.3.1 Cytokine network

The establishment of a cell culture system for the clonal development of hematopoietic cells made it possible to discover the proteins that regulate cell viability, growth and differentiation of different hematopoietic cell lineages and the molecular basis of normal and abnormal development in blood-forming tissues. These regulators include colony stimulating factors (CSFs) and interleukins (ILs), now called cytokines (Muench et al., 1992). Different cytokines can induce cell viability, multiplication and differentiation, and hematopoiesis is controlled by a network of cytokine interactions (Kiss et al., 2004). This multigene network includes positive regulators such as CSFs and ILs (Ido et al., 1992) and negative regulators such as transforming growth factor beta and tumor necrosis factor (Hartwig et al., 2001). The cytokine network which has arisen during evolution allows considerable flexibility depending on which part of the network is activated and the ready amplification of response to a particular stimulus. CSFs are cytokines that stimulate the proliferation of specific pluripotent stem cells of the bone marrow (Slanicka et al., 1998). The CSFs and ILs induce cell viability by inhibiting programmed cell death (apoptosis).

Stem cells are unspecialized precursor cells that have the unique ability to self-renew and generate additional stem cells as well as to differentiate into various progenitor cells in response to appropriate signals. It is well recognized that several growth and differentiation factors are responsible for shaping the destiny of stem cells. Self renewal and differentiation of hematopoietic stem cells is regulated by the hematopoietic microenvironment. The microenvironment of a cell plays an important role in the differentiation of individual bone marrow cells. Further differentiation of cells into one of several lineages critically depends on the nature of factors acting on these cells at a particular time and at a particular concentration. The bone marrow stroma contains many different cell types, including macrophages, fibroblasts, endothelial cells, smooth muscle cells, T-lymphocytes, monocytes etc. These cells, in combination with components of extracellular matrix and basement membranes, form

the so-called hematopoetic inductive microinvironment which maintains the functional integrity of this complex system of resident and circulating cells. Cells of the hematopoietic microenvironment show low or no detectable cell growth and are believed to be in the G0 phase of the cell cycle.

Many GFs are produced by stromal cells. They can be found both in membrane-bounded and soluble form. Membrane-bounded GFs on the surface of the stromal cells have an important role in the inductive microenvironment. E.g. To describe a functional analysis of the membrane-bound and soluble stem cell factor (SCF) within the context of stroma cocultures with hematopoietic cells, it was shown that transmembrane SCF is essential for long term growth, whereas soluble SCF supports only short term proliferation of stroma dependent hematopoietic cells (Verfaillie , 1993).

Hematopoietic stem cells (HSCs) proliferate and differentiate throughout the life cycle to produce lymphoid and myeloid cell types. Citokines act in a stepwise manner inducing proper maturation. Interleukin-3 (multi-CSF) and IL-6, stem cell factor (SCF) and Flt-3 ligand have been used as potential candidates for unspecific hematopoietic stimulation. They act early, possibly even at the level of the pluripotent stem cell, to induce formation of the nonlymphoid cells (erythrocytes, monocytes, granulocytes neutrophils, eosinophils, basophils and megakaryocytes) and lymphoid cells.

During hematopoiesis some more specific cytokines control differentiation and maturation of the committed cells in hematopoietic cell lines as erythropoietin, GM-CSF, G-CSF and thrombopoietin. Paralelly in cytokine network there are many synergistic effects and cytokines althougether regulate this process. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that regulates the survival, proliferation and differentiation of progenitor cells of neutrophilic and eosinophilic granulocytes, macrophages and megakaryocytic cells. In addition, it stimulates the functional activity of mature granulocytes and macrophages. GM-CSF synergises with erythropoetin in the proliferation of erythroid and megakaryocytic progenitor cells. M-CSF and G-CSF act still later, M-CSF promotes the formation of monocytes and G-CSF stimulates the proliferation and differentiation of hematopoietic progenitor cells, committed to the neutrophil/granulocyte lineage in a dose-

dependent manner. Differentiated neutrophilic granulocytes are functionally activated by G-CSF. G-CSF synergises with some other cytokines, including GM-CSF and IL4. GM-CSF and G-CSF are required, for example, to develop neutrophilic colonies in vitro. The concerted action of G-CSF and erythropoietin is required to support the growth of mixed colonies of the early erythroid progenitors.

1.3.2.Role of insulin in hematopoiesis

Insulin and insulin-like growth factors are early-acting growth factors for hematopoietic stem and progenitor cells. They are widely used for hematopoietic cell cultures to support colony formation of hematopoietic progenitor cells alone or together with some other growth factors. In spite of this there is no documentation of insulin's effects regarding hematopoiesis *in vivo*. In vivo effects of insulin are hindered by effects of insulin like growth factors (IGF 1,2) and insulin's numerous metabolic effects. It appeared that it could enhance the survival of the cells, significantly increased the number of all progenitors and doubled both the granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) recovered from CD34+ early progenitor hematopoietic cells *ex vivo* when compared to their insulin-free counterparts (Ratajczak et al., 1998). It has been demonstrated that IGF-I has a strong stimulatory effect on erythropoiesis.

In our work we studied effect of insulin on granulopoiesis *in vivo* in mice. However the use of insulin *in vivo* is not considered practical due to insulin's abundant metabolic effects e.g. hypoglycemia, therefore it is much more convenient to use an agent with same property and more moderate effect such as insulin sensitizers e.g. rosiglitazone. Therefore in our work we try to evaluate its possible myeloprotective effects as according to our hypothesis based on the findings insulin's protective effects *in vivo*, it could prevent anticancer drug induced myelotoxicity by increasing the host's insulin sensitivity. Rosiglitazone a thiazolidinedione insulin sensitizer developed for therapy of non-insulin dependent diabetes mellitus. (Vamecq and Latruffe, 1999) Its use increases the insulin sensitivity of the host and therefore increasing the positive effects of insulin.

1.4. Insulin sensitizers

Insulin resistance is the major cause in Non Insulin Dependent Diabetes Mellitus (NIDDM). It underlines the pathogenesis of hyperglycemia and cardiovascular disease in most people with type 2 diabetes. Thiazolidinediones, used for the treatment of diabetes mellitus type 2, counter insulin resistance by different cellular mechanisms (Bailey, 2005).

Thiazolidinediones modulate gene expression by binding to nuclear transcription factor, peroxisome proliferator-activated receptor-gamma. Peroxisome proliferator-activated receptor-gamma is expressed in several tissues, therefore, thiazolidinediones have biological effects on multiple organ systems.

This group of drugs, activators of peroxisome proliferator-activated receptors namely PPARγ, have emerged as an important class of drugs in the treatment of type II diabetes mellitus. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor isoforms with key roles in the regulation of lipid and glucose metabolism. Synthetic ligands for PPAR γ (and PPARα) have effects of promoting insulin sensitization in the context of obesity. Recent evidence suggests that activation of PPAR δ might produce similar effects. Both PPARγ and PPARα have also been shown to produce selected anti-inflammatory effects and to reduce the progression of atherosclerosis in animals (α and γ) or in humans (α). Mechanisms underlying insulin-sensitizing effects are complex. For PPARγ, direct effects on adipose tissue lipid metabolism with secondary benefits in liver and/or muscle (lipid levels and insulin signaling) have been implicated. For PPARα, accelerated lipid catabolism may contribute to reduced muscle or liver 'steatosis'. Anti-inflammatory mechanisms as contributors to the beneficial metabolic effects of PPAR activation are also worth considering.

Cardiovascular disease is significantly increased in patients with type 2 diabetes mellitus. Because of positive effects on glucose homeostasis, lipid metabolism, proteins involved in all stages of atherogenesis PPARα (fibrates) and PPAR-γ (glitazones) agonists are good candidates to reduce cardiovascular disease, more precisely in subjects with metabolic syndrome or type 2 diabetes mellitus. PPAR-γ agonists (glitazones) have not only beneficial

effects on glucose homeostasis, by increasing insulin sensitivity and reducing blood glucose level but also on lipid metabolism by elevating plasma HDL-cholesterol, decreasing free fatty acids and the number of small dense LDL particles, and for pioglitazone by reducing plasma triglycerides. Furthermore, they diminish vascular inflammation and vasoconstriction, inhibit monocyte chemotaxis, proliferation and migration of smooth muscle cells, in the vascular wall and decrease the production of adhesion molecules and metalloproteinases. PPARs γ agonists (glitazones) have been shown to reduce the development of atherosclerotic lesions in rats. Although the in vivo mode of action of PPAR-γ activators is not well understood, PPAR-γ is highly expressed in adipocytes and macrophages, suggesting these cells could be important targets. Paradoxically, however, PPAR-γ activators increase the expression of the proatherogenic molecule CD36 in cultured macrophages. As PPAR-γ nuclear receptors belonging in the steroid receptor family frequently form heterodimers with RXR retinoid receptors, maybe that they play a role in differentiation of these cells.

Rosiglitazone possess beneficial effects on other cardiovascular risk factors associated with the insulin resistance syndrome. Thus, were shown to decrease blood pressure, enhance myocardial function and fibrinolysis, as well as possess anti-inflammatory and other beneficial vascular effects. (Diamant and Heine, 2003).

2. AIMS

Insulin is an early-acting growth factor for hematopoietic progenitor cells assisting their proliferation and promotes their survival. It is commonly used in cell cultures. Insulin could be a possible protector of bone marrow progenitor cells against 5-fluorouracil (5-FU)-induced myelotoxicity bearing significant implications if able to demonstrate a considerable enhancement in restoration of stem cells and facilitate the recuperation phase after injury. In spite of insulin being widely used for hematopoietic cell cultures to support colony formation of hematopoietic progenitor cells, there is no documentation of its effects regarding hematopoiesis *in vivo.* Since insulin has many metabolic effects and can easily produce

hypoglycemia it is much more convenient to use an agent with same property and more moderate effect such as insulin sensitizers e.g. rosiglitazone. Since in vivo bone marrow protection is more advantageous, we also tested if rosiglitazone, a thiazolidinedione, could prevent anticancer drug induced myelotoxicity by increasing the host's insulin sensitivity.

The present work was initially concerned with the possibility of insulin and an insulin sensitizer namely rosiglitazone alleviating myelotoxicity induced by anticancer drug, 5-fluorouracil (5-FU), in mice. This myeloprotective effect of rosiglitazone is believed to possibly exist due to increasing insulin sensitivity of recovering cells. Primarily we examined the regeneration response of the 5-FU exposed bone marrow in mice that were pre-treated with rosiglitazone of various doses, to find out whether a more intensified bone marrow regeneration would be observed or not? What is the effect of rosiglitazone on the damaged bone marrow as a function of time? Would the recovering proliferation start earlier? If so when in the time line would be the highest intensity and how much longer do the non pre-treated group will take to reach the same level of regeneration. Also whether rosiglitazone has a direct influence on bone marrow cells was examined both in murine and human cell cultures. Using a PPARγ antagonist we investigated whether the observed protection connected to PPARγ receptorial effects of rosiglitazone. The goals of our experiments are summarized below.

Our aims were to investigate:

1. In vivo effects of insulin on granulopoiesis in mice with bone marrow damaged by a cytostatic agent, compared to same effects in healthy bone marrow.

2. Whether rosiglitazone has similar effects on normal and damaged bone marrow .

3. If rosiglitazone is able to protect more granulocyte-macrophage progenitor cells during bone marrow damage, and how it can influence the recovery of bone marrow as a function of time.

4. Whether rosiglitazone is able to mitigate the severity of neutropenia during the recovery after cytostatic drug-induced damage.

5. Whether rosiglitazone protects granulocyte-macrophage progenitor cells in bone marrow damaged by repeated doses of 5-FU.

6. Whether an insulin sensitizer drug, namely rosiglitazone has any effect on plasma insulin and glucose levels.

7. Whether rosiglitazone has any direct effects on progenitor cells of the murine bone marrow *in vitro* and are there any connection with its PPARγ receptorial effects.

8. Whether rosiglitazone has any direct effects on human stem and progenitor cells originated from peripheral blood of patients after their mobilization from bone marrow before autolougous peripheral blood stem cell transplantation.

3. Materials and methods

The present experiments conform to the European Community guiding principles for the care and use of laboratory animals. The experimental protocol has been approved by the Ethics Committee for Animal Research, University of Debrecen (11/2002 DEMAB).

3.1. Animals

Mice: 10-11 weeks old female offspring of C57black x DBA mice – weight: 20-35 g obtained from the National Institute of Oncology, Budapest, Hungary. Animals were housed in an animal room with 3-5 mice per pen, and were given standard laboratory food and water ad libitum. Keeping conditions were controlled by the Regional Ethical Committee for Animal Experiments, conforming to the Standards of the European Union. Body weight of mice was measured twice, prior to the experiment and on the day of their extermination. The experimental protocol has been approved by the Ethics Committee for Animal Research, University of Debrecen (11/2003 DEMAB).

3.2. Patients

Management of patients conformed to the Helsinki Declaration. Informed consent and study design were approved by Regional Human Ethics Committee, University of Debrecen (55/2003). The three patients with hematological malignancies were waiting on autologous peripheral stem cell transplantation in Department of 2nd Internal Medicine, University of Debrecen. During clinical remission progenitor cells were mobilized from their bone marrow. Samples were obtained from the rest of the separated frozen stem cell suspension at the time of transplantation.

3.3 Materials

5-FU : Fluorouracil-TEVA, Pharmachemie, Haarlem, Netherlands) dissolved in 0.9% NaCl.

Rosiglitazone: Avandia, GlaxoSmithKlein, Brentford, United Kingdom

Stock solution : It was prepared by acidic Aqua destillata and grinded Avandia tablets (GlaxoSmithKlein, Brentford, United Kingdom). The 4 mg-contained tablet was grinded and suspended in 6.6 ml vehicle to obtain 0.6 mg/ml of rosiglitazone stock solution. 2 and 4 times additionally dilutions were also used for different groups. pH of the vehicle was about 2-3.

Rosiglitazone for in vitro experiments: A gift from Rich Heyman, X-Ceptor Therapeautics Inc., San Diego, CA, USA

Insulin : Human regular insulin,(NOVO, Nordisk, Copenhagen) used for determination of insulin sensitivity

Insulin: (used for demonstrating the effects on colony formation of granulocyte-macrophage progenitor cells) Insulin Monotard HMge, Novo Nordisk, Bagsvaerd, Denmark)

Stock solution : It was prepared by Physiologic NaCl and insulin Insulin Monotard HMge, Novo Nordisk, Bagsvaerd, Denmark),at 40 U /ml concentration. After dilution samples were additionally diluted 1.5 and 3 times for application to different groups.

Peroxisome-proliferator-activated receptor-gamma (PPARγ) antagonist GW9662, a gift from T. M. Willson, GlaxoSmithKline, Research Triangle Park, NC.

Stock solutions were prepared freshly before each experiment.

3.4. Study design of in vivo experiments

3.4.1. Myeloprotective effects of insulin

Mice were randomly assigned into 8 groups. Groups 1-4 served as different controls. Vehicles of insulin and 5-FU, 4 or 6 U/kg of insulin and/or 70 or 100 mg/kg of 5-FU were administered to mice in groups 1, 2,3 and 4 respectively. Animals in groups 5-6 received subcutaneous injection of 4 or 6 U/kg insulin followed by a single dose of 5-FU 70mg/kg. Animals in groups 7-8 received 4 or 6 U/kg subcutaneous injection of insulin followed by a single dose of 5-FU 100mg/kg. Insulin was delivered by subcutaneous gavage for five days, 96, 72, 48, 24 and 1 hour before the single intraperitoneal dose of 70 or 100 mg/kg 5-FU (Fluorouracil-TEVA, Pharmachemie, Haarlem, Netherlands) dissolved in 0.9% NaCl.
The experiment was repeated 3 times.

Bone marrow function were evaluated following the injection of 5-FU in separate sets of animals. The peripheral leukocyte count and absolute neutrophil count (ANC) were determined from samples of retro-orbital sinus blood. Cellularity of femoral bone marrow was calculated from bone marrow cell counts and volumes of the samples, the frequency of CFU-GM progenitors was established from the soft agar cultures. Total CFU-GM content of the femur was calculated (cellularity x frequency of CFU-GM.

1. Table. *Treatment schedule to evaluate myeloprotective effects of insulin*

No.	1-5 days	1-5 days	5 day	5 day
Groups(8x3 mice)	Aqua dest	Subcutaneous insulin	Phys NaCl	ip. 5-FU
1. Control	+	_	+	_
2. 6 U/kg insulin	_	+	+	_
3. 70 mg/kg 5-FU	+	_	_	+
4. 100mg/kg 5-FU	+	_	_	+
5. 4U/Kg insulin +70 mg/kg 5-FU	_	+	_	+
6. 6U/Kg insulin +70 mg/kg 5-FU	_	+	_	+
7. 4 U/Kg insulin +100 mg/kg 5-FU	_	+	_	+
8. 6 U/Kg insulin +100 mg/kg 5-FU	_	+	_	+

3.4.2. Determining insulin sensitivity and insulin plasma levels

Mice were randomly assigned into 4 groups. Groups 1 served as control. Treated only by vehicle. Rosiglitazone of 1.5 or 3 or 6 mg/kg concentration was applied for 5 days orally to groups 2,3 and 4.

On the 5th day insulin sensitivity was determined. The animals were anesthetized with an initial interaperitoneal dose of 50-mg/kg thiopental-sodium that was repeated as needed. For hyperinsulinaemic euglycaemic glucose clamping two venous catheters and one arterial cannula were placed in the two external jugular veins, and the right carotid artery respectively. Human regular insulin was infused at a constant rate (20mU/kg, NOVO, Nordisk, Copenhagen) via one of the venous catheters over 120 minutes to yield steady state plasma insulin immunreactivity of 100±5 μU/ml. The glucose infusion rate (mg/kg/min) during the steady state characterized insulin sensitivity (DeFronzo et al., 1979). In the steady state additional blood samples were taken for plasma insulin determination three times at 10 minutes intervals.

Similarly we determined insulin sensitivity together with the *insulin blood levels* in 6 groups treated with vehicle, 3 and 6 mg/kg of rosiglitazone,100 mg/kg of 5-FU,3 or 6 mg/kg of rosiglitazone together with 5-FU, during the course of other experiment.

2. Table. *Treatment schedule and animal groups*

Groups	Vehicle	Rosiglitazone
	5days	5days
1. Control	+	_
2. 1.5mg/kg rosiglitazone	_	+
3. 3mg/kg rosiglitazone	_	+
4. 6mg/kg rosiglitazone	_	+

3.4.2.1. Hyperinsulinemic euglycemic glucose clamp

The hyperinsulinemic euglycemic glucose clamp (HEGC) was preformed as originally described by DeFronzo et al.. Continuous insulin infusion (Humulin R®, Eli Lilly, Indianapolis, IN) at constant rate of 15 mU/kg/min for 120 min and 20% (w/v) glucose infusion were adjusted to maintain the euglycaemic (5.5±0.5 mol/l) blood glucose concentration. Blood samples (10 μl) were obtained from the carotid artery for determination of blood glucose concentration by means of a glucometer (Roche Paris, France), before and at 5 min intervals during the first 80 min of the clamp and at 10 min intervals during the last 40 min of the clamp. Immediately before the insulin infusion was started an arterial blood sample (0.5 ml, in 20 μl EDTA, 10 μl Gordox) was collected, centrifuged, and the plasma aliquoted, frozen and stored at -70 ºC for subsequent determinations of plasma insulin levels. Steady state insulin levels were also determined by the same way as described above at 100 and 120 min, respectively. The glucose infusion rate (GIR; mg/kg/min) need to maintain the euglycaemic blood glucose target concentration was used to characterize peripheral insulin sensitivity.

The animals were anaesthetized with an initial intraperitoneal dose of 50 mg/kg thiopenthal-sodium that was repeated as needed. For hyperinsulinaemic euglycaemic glucose clamping two venous catheters and one arterial cannula were placed in the two external jugular veins, and the right carotid artery respectively.
Human regular insulin was infused at a constant rate (20mU/kg, NOVO, Nordisk, Copenhagen) via one of the venous catheters over 120 minutes to yield steady state plasma insulin immunreactivity of 100±5 μU/ml. Blood samples of 0.2 ml were taken from the arterial cannula for blood glucose concentration measurements at 10 minutes intervals. Blood glucose concentration was maintained constant (5.5±0.5 mmol/l) by variable rate of glucose infusion via the second venous cannula. Stable blood glucose level of 30 minutes duration was defined as steady state. The glucose infusion rate (mg/kg/min) during the steady state characterized insulin sensitivity (DeFronzo et al., 1979). In the steady state additional blood samples were taken for plasma insulin determination three times at 10 minutes intervals.

3.4.2.2. Insulin blood levels

Plasma glucose concentrations were determined in blood samples taken from the retroorbital plexus of the mice using Accu-Chek (Roche Diagnostics, Mannheim, Germany). Plasma insulin level was measured by means of radioimmunoassay (RIA) using commercially available kits (RK 400 M, Institute of Isotopes, Budapest, Hungary). Both intra- and inter-assay variations were lower than 5%.

3.4.3. Myeloprotective effects of rosiglitazone

3.4.3.1. Effect of rosiglitazone on bone marrow function determined on the 2nd day after damage

To screen whether rosiglitazone has any myeloprotective effect first we used the 2nd day after 5-FU injection for evaluating changes in bone marrow function.

Mice were randomly assigned into 6 groups. Groups 1-3 served as different controls. Vehicles of rosiglitazone and 5-FU, or 6 mg/kg of rosiglitazone or 100 mg/kg of 5-FU were administered to mice in groups 1, 2 and 3 respectively. Animals in groups 4-6 received 3 or 6 mg/kg rosiglitazone (Avandia, GlaxoSmithKlein, Brentford, United Kingdom) followed by a single dose of 5-FU. Rosiglitazone diluted in distilled water was delivered by oral gavage for five days, 96, 72, 48, 24 and 1 hour before the single intraperitoneal dose of 100 mg/kg 5-FU (Fluorouracil-TEVA, Pharmachemie, Haarlem, Netherlands) dissolved in 0.9% NaCl.

The experiment was repeated several times were the animals were terminated on the 2nd day, by cervical dislocation after application of 100 mg/kg 5-FU, in order to obtain bone marrow samples from femora. Bone marrow function and insulin sensitivity were evaluated following the injection of 5-FU in separate sets of animals.

Before terminating the animals, samples were obtained from retro-orbital plexus of mice and measured blood cell count.

The peripheral leukocyte count and absolute neutrophil count (ANC) were determined from samples of blood. Cellularity of femoral bone marrow was calculated and bone marrow cells were cultured under special conditions to determine CFU-GM progenitor cells. Degree of bone marrow damage was indicated by the changes in cellularity, frequency of CFU-GM and the total CFU-GM content of femoral bone marrow.

Total CFU-GM content of the femur was calculated (cellularity x frequency of CFU-GM). Insulin sensitivity was determined by hyperinsulinaemic euglycaemic glucose clamp.

3. Table. Treatment schedule and animal groups

Groups	5^{th} day	1-5. day
	ip. Application	po. Application
1.Control	Phys.NaCl	Vehicle
2.rosiglitazon 6 mg/kg	Phys. NaCl	Rosiglitazone
3.5-FU 100 mg/kg	5-FU	Vehicle
4.5-FU +rosiglitazon 3 mg/kg	5-FU	Rosiglitazone
5.5-FU +rosiglitazon 6 mg/kg	5-FU	Rosiglitazone

3.4.3.2. Effect of rosiglitazone pre-treatment on vulnerability of CFU-GM progenitors to 5-FU-induced toxicity.

To evaluate whether rosiglitazone could preserve more bone marrow progenitor cells during damage caused by a cytostatic agent, mice were randomly assigned into 4 groups. The first three groups served as different controls. Vehicles of rosiglitazone and 5-FU, or 6 mg/kg of rosiglitazone or 100 mg/kg of 5-FU alone were administered to the mice. The remainder was pretreated with rosiglitazone (Avandia, GlaxoSmithKlein, Brentford, United Kingdom) in 6 mg/kg doses followed by a single dose of 5-FU. Rosiglitazone diluted in distilled water was

delivered by oral gavage for five days, 96, 72, 48, 24 and 1 hour before the single intraperitoneal dose of 100 mg/kg 5-FU (Fluorouracil-TEVA, Pharmachemie, Haarlem, Netherlands) dissolved in 0.9% NaCl.

Bone marrow functions were evaluated one-hour after the injection of 5-FU in separate sets of animals. Cellularity of femoral bone marrow was calculated from bone marrow cell counts and volumes of the samples. The frequency of CFU-GM progenitors was established from the soft agar cultures. Total CFU-GM content of the femur was calculated (cellularity x frequency of CFU-GM).

4. Table. Treatment schedule and animal groups

Groups	5^{th} day	1-5. day
	ip. Application	po. Application
1.Control	Phys.NaCl	Vehicle
2.rosiglitazon 6 mg/kg	Phys. NaCl	Rosiglitazone
3. 5-FU 100 mg/kg	5-FU	Vehicle
4. 5-FU +rosiglitazon 6 mg/kg	5-FU	Rosiglitazone

3.4.3.3. Effect of rosiglitazone on recovery of bone marrow after a single dose of 5-FU.

Mice were randomly assigned into 6 groups. Groups 1-3 served as different controls. Vehicles of rosiglitazone and 5-FU, or 6 mg/kg of rosiglitazone or 100 mg/kg of 5-FU were administered to mice in groups 1, 2 and 3 respectively. Animals in groups 4-6 received 3 or 6 mg/kg rosiglitazone (Avandia, GlaxoSmithKlein, Brentford, United Kingdom) followed by a single dose of 5-FU. Rosiglitazone diluted in distilled water was delivered by oral gavage for five days, 96, 72, 48, 24 and 1 hour before the single intraperitoneal dose of 100 mg/kg 5-FU (Fluorouracil-TEVA, Pharmachemie, Haarlem, Netherlands) dissolved in 0.9% NaCl.
The experiment was repeated several times were the animals were terminated at different time

points in order to compare their response to rosiglitazone against 5-FU.

Experiments were repeated according to the different studied days after the damage caused by a single 5-FU dose to observe dynamics of recovery and 3 independent experiments were done in each studied time point. On the studied days after the single 100 mg/kg of 5 –FU dose blood samples were obtained from retroorbital plexus of mice and measured for blood cell count. Afterwards animals were exterminated by cervical dislocation in order to obtain bone marrow samples from femora. Cellularity of femoral bone marrow was calculated and bone marrow cells were cultured under special conditions to determine CFU-GM progenitor cells. Degree of bone marrow damage was indicated by the changes in cellularity, frequency of CFU-GM and the total CFU-GM content of femoral bone marrow.

3.4.3.4. Effect of rosiglitazone on damage caused by repeated doses of 5-FU.

To see whether rosiglitazone has myeloprotective effect in bone marrow damage caused by repeated doses of 5-FU mice were randomly assigned into 6 groups. Mice were treated daily for 7 days. Rosiglitazone in 6 mg/kg doses and 5-FU were simultaneously administered to the mice in the combination treatment groups with one-hour intervals between the doses. The control mice in the 1st group were treated with vehicles of rosiglitazone and 5-FU. The mice in the 2nd group were administered rosiglitazone alone. In groups 3 and 4, 25 mg/kg of 5-FU was used after the rosiglitazone administration, while in groups 5 and 6, 50 mg/kg was the chosen dose for 5-FU. Bone marrow function was assessed in the same manner as previously described. After sacrifice of the animals, bone marrow samples were obtained from their femurs. Bone marrow functions were evaluated one-hour after the injection of 5-FU in separate sets of animals. Cellularity of femoral bone marrow was calculated from bone marrow cell counts and volumes of the samples. The frequency of CFU-GM progenitors was established from the soft agar cultures. Total CFU-GM content of the femur was calculated (cellularity x frequency of CFU-GM).

5. Table. Treatment schedule and animal groups

No.	1-7 days	1-7 days	7 day	7day
Groups(6)	Vehicle	p.o rosiglitazone	phys NaCl	ip. 5-FU
1. Control	+	_	+	_
2. 6mg/kg rosiglitazone	_	+	+	_
3. 25mg/kg 5-FU	+	_	_	+
4. 6mg/kg rosiglitazone +25mg/kg 5-FU	+	_	_	+
5. 50 mg/kg 5-FU	+	_	_	+
6. 6mg/kg rosiglitazone +50 mg/kg 5-FU	_	+	_	+

3.5. Biological samples

3.5.1. Blood samples

Blood was obtained from the retroorbital plexus immediately before their extermination for counting white blood cell numbers and blood smears , in order to determine the white blood cell count (WBC) and absolute neutrophil granulocyte count (ANC) =(WBC * frequency of neutrophils).

These values were counted in haemocytometer, the frequency of neutrophil granulocytes was determined by Total white blood cell count differential count of 200 cells from blood smears stained with Wright-Giemsa.

In determining insulin plasma levels, intracardial blood was used. Reminder of the procedure followed the same rout as previously described.

3.5.2 Bone marrow samples

Bone marrow was obtained from the femora of the mice after their extermination by cervical dislocation. One of the femurs was removed and the bone marrow cells were aseptically washed out. Single cell suspensions were prepared suspending them in serum-free McCoy's 5A medium (GIBCO) through a thin needle by a syringe. Femoral bone marrow was obtained after the extermination of the mice. From bone marrow cell suspensions cell numbers (BMC) were counted in a hemocytometer to determine the cellularity of the bone marrow, which was expressed as nucleated cells per femur.

3.6 Bone marrow soft gel cultures (CFU-GM colony assay).

Soft-gel cell cultures were prepared from the murine bone marrow cell suspensions and after culturing period the number of CFU-GM (granulocyte-macrophage progenitor cells) was determined by counting colonies under stereomicroscope.

Soft agar cultures were made by using inocula of 10^5/ml bone marrow cells in petri dishes (Greiner, Nürtingen, Germany)murine bone marrow cells were grown in McCoy's 5A modified medium (GIBCO Grand Island NY USA) supplemented with amino acids, Na pyruvate, $NaHCO_3$ and antibiotics (streptomycin, penicillin) according to Pike and Robinson (1970) as well as with 0.3% agar (Ionagar No2, Oxoid, London, Great Britain), as well as with 20-25% horse serum. The best batch of serum was selected in preliminary experiments from several samples supplied by the manufacturer. The source of colony-stimulating factor was the medium conditioned by WEHI-3B cells (WEHI-3B-CM), produced and tested in our laboratory and used at a concentration necessary for the growth of maximum number of colonies. WEHI-3B-CM contains a variety of growth factors. Three parallel petri dishes (Greiner, Nürtingen, Germany) containing the cultures were incubated for 7 days in a humidified atmosphere containing 5% CO_2 in CO_2 incubator (JOUAN Co, France).

3.7. In situ and citospin preparations

Morphology of cells in the colonies was evaluated in *in situ* touch and cytospin preparations in order to assess whether the colonies were of normal myeloid precursor cells (granulocyte-macrophage colony forming unit; CFU-GM). Slides were stained by May Grünwald Giemsa.

3.8. Evaluation of bone of bone marrow function

Colonies, defined as groups of at least 50 cells, were counted under a dissecting microscope (SZ6045; Olympus, Hamburg, Germany) at the end of the incubation period. Colony numbers represent the mean of 3-4 parallel cultures. Cellularity of femoral bone marrow was calculated from bone marrow cell counts and volumes of the samples, the frequency of CFU-GM progenitors was established from the soft methylcellulose cultures.Numbers of colonies grown from 10^5 bone marrow cells showed the intensity of regeneration of CFU-GM pool after the bone marrow damage caused by cytostatic agent. The CFU-GM content of femur (CFU-GM pool) was calculated with the help of the colony numbers and cellularity and was expressed as CFU-GM per femur.

1. Cellularity = Bone Marrow Cell Count x volume of bone marrow cell suspension means the total nucleated cell number in the femora of mice

2. Frequency of CFU-GM = number of colonies of CFU-GM/ 10^5 bone marrow cells (number of granulocyte-macrophage colony forming units/ 10^5 bone marrow cells)

3. Total CFU-GM content of the femur was calculated : cellularity x frequency of CFU-GM.

3.8. Study design of in vitro experiments

3.9.1. Effects of rosiglitazone on damage of murine CFU-GM progenitors *in vitro*

In vitro effects of rosiglitazone on murine CFU-GM cells were studied in methylcellulose cultures. The femoral bone marrow cell suspension was divided into six portions and cells were grown in the presence of rosiglitazone at a concentration of 1μM in the 2nd, 5th and 6th series. On the 5th day of the culturing period 5-fluorouracil was added to the 4th, 5th and 6th series cultures in a 1 mg/l final concentration

3.9.2. Effects of PPARγ antagonist on rosiglitazone-induced protection *in vitro*

In vitro effects of rosiglitazone on murine CFU-GM cells were studied in methylcellulose cultures. The femoral bone marrow cell suspension was divided into six portions and cells were grown in the presence of rosiglitazone at a concentration of 1μM in the 2nd, 5th and 6th series. On the 5th day of the culturing period 5-fluorouracil was added to the 4th, 5th and 6th series cultures in a 1 mg/l final concentration. In the 3rd and 6th series of cultures GW9662, a peroxisome-proliferator-activated receptor-gamma (PPARγ) antagonist (gift from T. M. Willson, GlaxoSmithKline, Research Triangle Park, NC) was also present from the beginning of the culturing period in a 5 μM concentration. Cells in the control cultures (1st series) were treated with vehicle in the same manner as the 1st series.

3.9.3. Effects of rosiglitazone on damage of human mobilized peripheral blood stem cells *in vitro*

To detect whether previous beneficial effects of rosiglitazone had a direct or an indirect influence on the hemopoietic cells, in vitro cultures were used. In our experimental system their differentiation into granulocyte-macrophage cells could be studied. Cells were cultured both in presence and absence of rosiglitazone. 5-FU was added to cultures later, on the 5th day.

3.10. Mobilization of bone marrow stem cells and leukapheresis

Patients with multiple myeloma were selected.Bone marrow stem cells were mobilized to the blood with a single 4 g/m^2 dose of Cytoxan (Brystol Myers Squibb Co., Princeton, New Jersey USA) and 48 MU granulocyte colony-stimulating factor (G-CSF, Neupogen, Hoffmann-La Roche Ltd., Basel, Switzerland) two times daily from the 3rd day until the 10-11th day. Mobilized peripheral blood stem cells were obtained by leukapheresis using Fresenius Com.Tec system (Fresenius Com.Tec GmbH Hamburg, Germany). Apheresis was initiated on the 10-11th day in the recovery phase after chemotherapy, if the $CD34^+$ cell count was higher than 20/□l in the blood. 2-3x10^8/kg mononuclear cells with 3-4x10^6/kg $CD34^+$ cells were obtained from the patients. Cells were resuspended in 100 ml IMDM with 1% human serum albumin, mixed slowly with equal volume of freezing solution containing 5 % DMSO in final concentration. The samples then were frozen by computer-controlling cryopreservating system (Cryomed Freezer, Thermo Forma, Marietta, Ohio, USA) at –190^{o}C using liquid nitrogen at the Cell Therapy Laboratory, University of Debrecen. Cells were thawed rapidly in a water bath maintained at 37 ^{o}C. The total volumen of the separated and thereafter thawed cells were used for the autologous transplantation and the rest cells are cultured in our experiments.

3.11. Colony formation of human mobilized peripheral blood stem cells

Methylcellulose (Methocel, 3000-5000 centipoise; FLUKA, Buchs, Switzerland) at 1.2% was used as the support matrix for semisolid cultures. McCoy's 5A modified medium was supplemented with amino acids, vitamins, Na-pyruvate, $NaHCO_3$, penicillin and streptomycin as well as with 5x10^{-5} M 2-mercaptoethanol (LOBA, Fischamend, Germany) and 20% FBS (Benk* et al., 2000). Mobilized peripheral blood stem cells were separated by Ficoll-Iodamide (Pharmacia, Uppsala, Sweden) gradient centrifugation at 1000g for 15 min (specific gravity, 1.077 g/ml). Mononuclear cells from the interphase were washed twice with McCoy's 5A medium containing 5% fetal bovine serum (GIBCO, Grand Island, NY, USA). Using 35 mm plastic petri dishes (Greiner, Nürtingen, Germany). In 1 ml volume of this medium 10^5 cells were plated and were incubated for 14 days at 37^{o}C in a humidified atmosphere containing

5% (v/v) CO_2. Cultures were seeded in triplicates or quadruplicates. Cytokines were added to the soft gel cultures just before plating in final concentrations of 300 mg/l for G-CSF (Genzyme, Cambridge, England) and of 100 mg/l for GM-CSF (Genzyme, Cambridge, England). Rosiglitazone was mixed to the medium and 5-FU was added to the cultures on the 5^{th} day of the 14-day-long cultural period. Colonies were counted under a dissecting microscope (Olympus, Hamburg, Germany). Colonies were defined as groups of at least 20 cells. Morphology of cells in the colonies was evaluated in in situ touch and cytospin preparations. Slides were stained by May-Grünwald-Giemsa or by conventional cytochemical reactions.

3.12. Statistical analysis

Data obtained from individual mouse were used for statistical analysis. Each haematologic and insulin sensitivity 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 $p<0.05$.

4. RESULTS

4.1 In vivo effects of insulin pre-treatment on hematopoiesis of healthy and damaged bone marrow

Serious bone marrow damage resulted from 5-fluorouracil in 70 and 100 mg/kg doses. The dose-dependent decrease in colony formation of CFU-GM progenitor cells is evident 2 days after 5-fluorouracil administration. When 6 U/kg of long-acting insulin was administered once a day for 5 days before the single 5-fluorouracil dose, the CFU-GM colony numbers grown from 10^5 mononucleated cells were significantly higher than in vehicle-pre-treated groups.(Fig1)

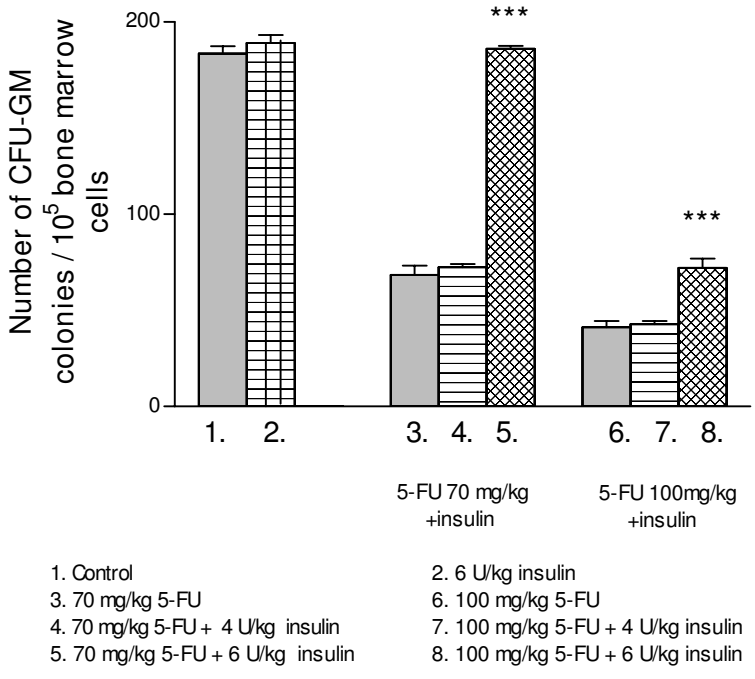

Fig. 1 *Insulin effects on bone marrow damage caused by 5-FU in mice.*
Influence of 5-day subcutaneous insulin pre-treatment in various doses on 5-fluorouracil-induced myelotoxicity in 70 and 100 mg/kg concentrations indicated by CFU-GM colony

formation. Cultures were grown in triplicates. Values are means ± S.E.M., n=13 in each group,*** $P<0.001$ compared to mice treated with 5- fluorouracil alone. Mice in control group were treated with vehicle.

4.2. Effect of rosiglitazone on insulin sensitivity in mice

Rosiglitazone is known as an insulin sensitizing drug. In these experiments we determined the dose range, which increased insulin sensitivity in mice.

Rosiglitazone applied at oral doses of 6 mg/kg resulted in a significant increase of the insulin sensitivity. The lower doses (1.5 and 3 mg/kg) and the vehicle were without effect. (Fig 2)

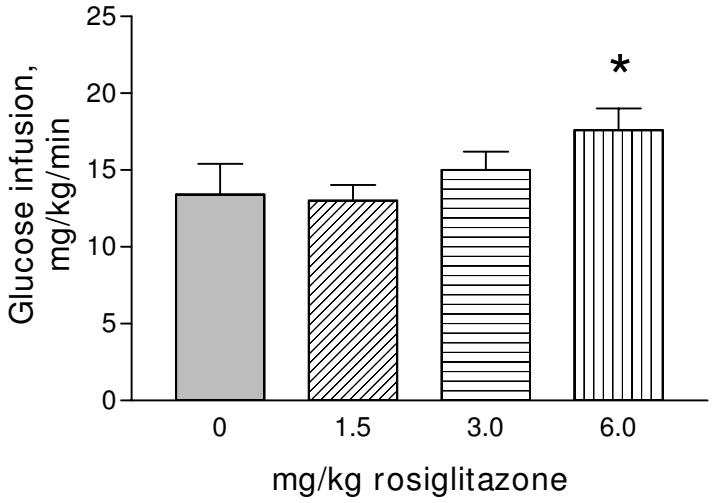

Fig 2. *Effects of rosiglitazone on insulin sensitivity*
Influence of rosiglitazone at 1.5 and 3, and 6 mg/kg on insulin sensitivity in presence of glucose infusion.

4.3. Effect of rosiglitazone pre-treatment on 5-fluorouracil-induced myelosuppression

Effect is determined from the comparisons of the 2nd day states of the bone marrow function after the damage caused by 5-FU in the pretreated and non-pretreated groups. 5-FU significantly reduced cellularity, CFU-GM colony number and content of the femoral bone marrow (P<0.001) The cellularity of the bone marrow did not differ in the rosiglitazone pre-treated groups and the 5-FU-treated group (Fig.3).

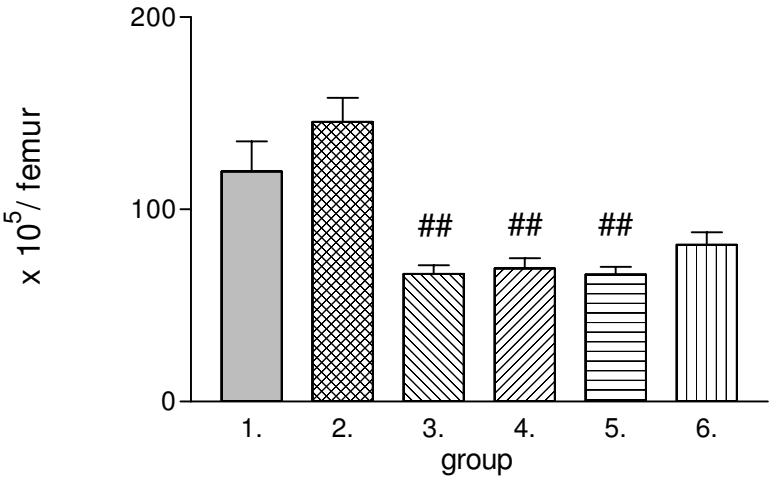

Fig 3. *Femoral bone marrow cellularity in the function of time.*

Influence of 5-day–long oral rosiglitazone pre-treatment in doses of 1.5, 3 and 6 mg/kg on recovery of the 5-fluorouracil-damaged bone marrow indicated by cellularity of the bone marrow. Days are numbered from the single intraperitoneal injection of 5- fluorouracil. Cultures were grown in triplicates. Values are means ± S.E.M., n=13 in each group **P*<0.05 , **P*<0.01 , **P*<0.001 compared to the control group treated with vehicle

Group 1: control, group 2: rosiglitazone-treated (6mg/kg), group 3: 5-FU-treated (100 mg/kg), group 4: rosiglitazone(1.5 mg/kg) + 5-FU-treated, group 5: rosiglitazone(3 mg/kg) + 5-FU-treated, group 6: rosiglitazone(6 mg/kg) + 5-FU-treated

The frequency of CFU-GM progenitors, indicated by the colony numbers grown from 10^5 bone marrow cell inoculates, were higher in samples obtained from mice treated with rosiglitazone and 5-FU than in mice treated with 5-FU alone. This effect of rosiglitazone was dose-dependent. Rosiglitazone in a dose of 1.5 mg/kg had no influence on colony numbers. Three and six mg/kg of rosiglitazone increased colony numbers by 2 ($p < 0.05$) and 2.5 fold ($p < 0.001$) respectively (Fig. 4).

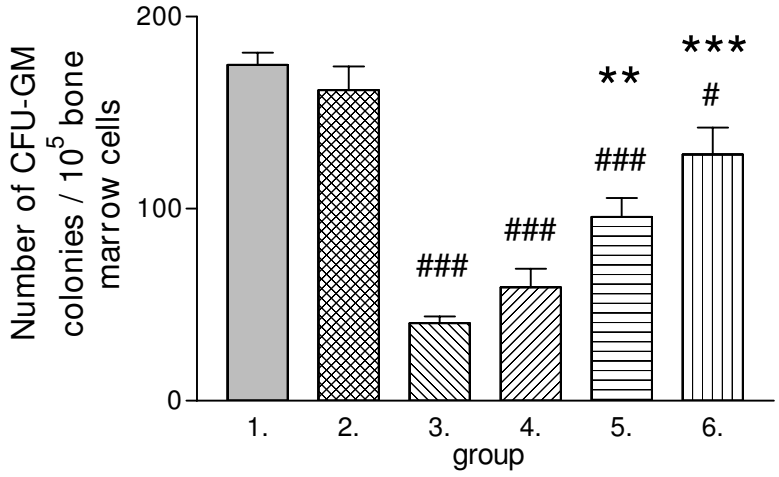

Fig 4. *Number of CFU-GM colonies on the 2nd day after a single dose of 5-FU.*
Influence of 5-day–long oral rosiglitazone pre-treatment in doses of 1.5, 3 and 6 mg/kg on recovery of the 5-fluorouracil-damaged bone marrow indicated by CFU-GM colony numbers of the bone marrow. Days are numbered from the single intraperitoneal injection of 5-fluorouracil. Cultures were grown in triplicates..Cultures were grown in triplicates Values are means ± S.E.M., n=13 in each group **P*<0.05 , **P*<0.01 , **P*<0.001 compared to the control group treated with vehicle. Group 1: control, group 2: rosiglitazone-treated (6mg/kg), group 3: 5-FU-treated (100 mg/kg), group 4: rosiglitazone(1.5 mg/kg) + 5-FU-treated, group 5: rosiglitazone(3 mg/kg) + 5-FU-treated, group 6: rosiglitazone(6 mg/kg) + 5-FU-treated

Proliferation of the increased number of progenitors result in an expansion of the CFU-GM pool and 6 mg/kg of rosiglitazone could significantly increase the CFU-GM content after the bone marrow damage ($p < 0.05$)(Fig.5).

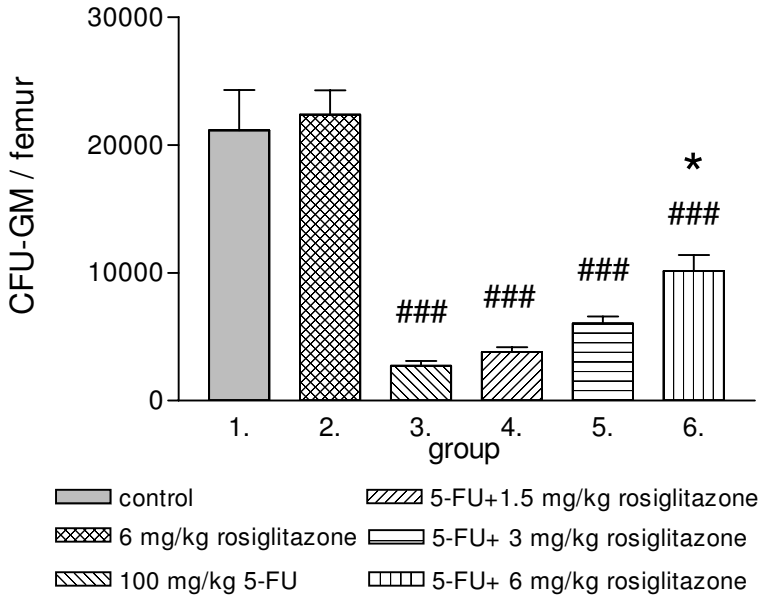

Fig 5. CFU-GM content in the bone marrow after a single dose of 5-FU.

Influence of 5-day–long oral rosiglitazone pre-treatment in doses of 1.5, 3 and 6 mg/kg on recovery of the 5-fluorouracil-damaged bone marrow indicated by CFU-GM content of the bone marrow. Days are numbered from the single intraperitoneal injection of 5- fluorouracil. Cultures were grown in triplicates..Cultures were grown in triplicates Values are means ± S.E.M., n=13 in each group *P<0.05 , *P<0.01 , *P<0.001 compared to the control group treated with vehicle.

4.4. Effect of rosiglitazone on recovery of absolute neutrophil cell counts (ANC) after a single dose of 5-FU

The toxic effect of 5-FU was also investigated in peripheral blood samples. Cytopenia originated from the lack of progenitors appears later in blood than it does in bone marrow.

The absolute neutrophil counts (ANC) were a somewhat lower in the 5-FU-treated group than in the control group, however this difference was not significant. Furthermore these values were all within the normal range in each group (Fig 6).

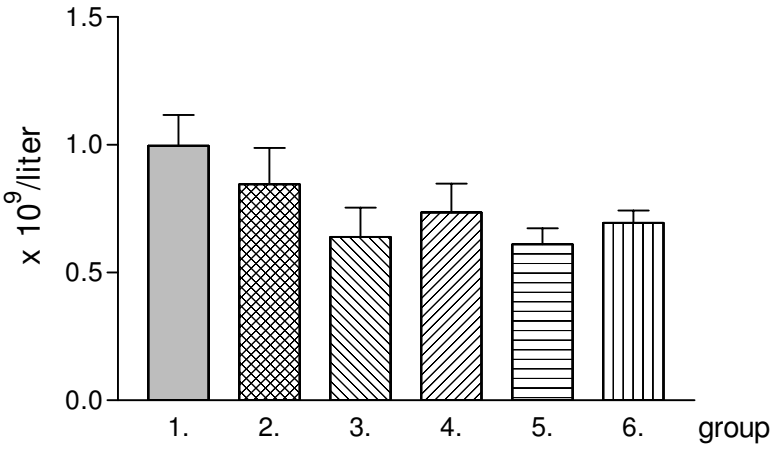

Fig 6. *Influence of 5-day oral rosiglitazone pre-treatment on absolute neutrophil count in periferal blood 2 days after a single dose of 5-FU.*

Group 1: control, group 2: rosiglitazone-treated (6mg/kg), group 3: 5-FU-treated (100 mg/kg), group 4: rosiglitazone (1.5 mg/kg) + 5-FU-treated, group 5: rosiglitazone (3 mg/kg) + 5-FU-treated, group 6: rosiglitazone (6 mg/kg) + 5-FU-treated

4.5. Effect of rosiglitazone pre-treatment on vulnerability of CFU-GM progenitors against to 5-FU-caused toxicity

Effect is determined from the comparisons of the states of the bone marrow function immediately after the damage caused by 5-FU in the pretreated and non-pretreated groups.

One hour after injection of a single intraperitoneal dose of 100 mg/kg 5-fluorouracil (5-FU) bone marrow function indicated by cellularity showed little difference between groups pre-treated with rosiglitazone and non pre-treated groups.(Fig 7)

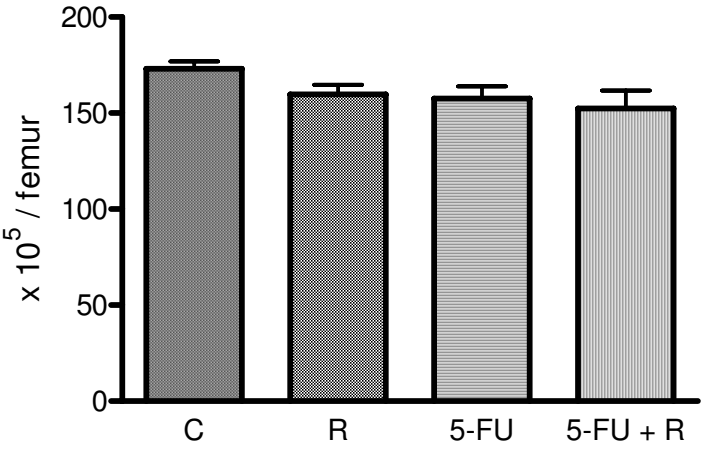

Fig 7. *Effect of rosiglitazon on femoral bone marrow cellularity after a single intraperitoneal injection of 5-FU.*

Influence of 5-day oral rosiglitazone pre-treatment in 6 mg/kg dose on bone marrow damage caused by a single 100 mg/kg intraperitoneal dose of 5-fluorouracil (5-FU) indicated by total cellularity of the bone marrow .Cultures were grown in triplicates. Values are means ± S.E.M., n=8 in each group. Mice treated with 5-FU alone. Mice in control group were treated with vehicle. Group C: control, group R: rosiglitazone-treated (6mg/kg), group 5-FU: 5-FU-treated (100 mg/kg), group 5-FU+R: rosiglitazone (6 mg/kg) + 5-FU-treated

The frequency of CFU-GM progenitors, indicated by the colony numbers grown from 10^5 bone marrow cell inoculates, was higher in samples obtained from mice treated with rosiglitazone for 5 days before the 5-FU dose than in mice treated with 5-FU alone. Comparing the bone marrow frequency between pretreated and non-pretreated mice, allowing only one hour for regeneration after 5-FU injection, groups receiving pretreatment showed a higher preservation of the CFU-GM pool in bone marrow. Furthermore the frequency of progenitor cells seemed to have improved in groups receiving pretreatment of rosiglitazone ($P<0.001$) (Fig 8).

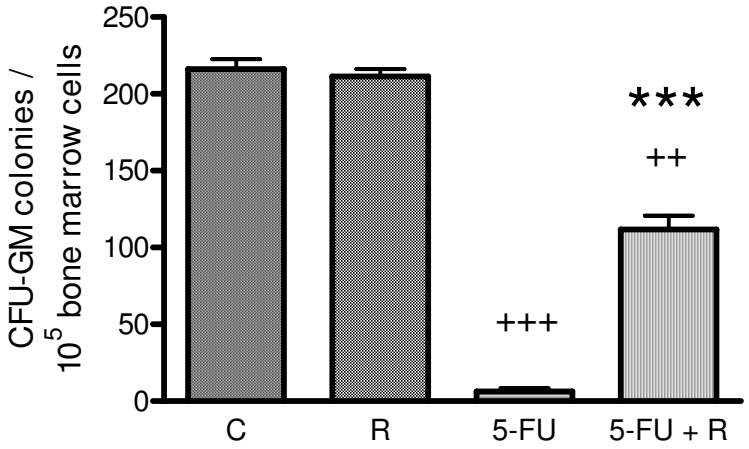

Fig 8. *Effect of rosiglitazon on CFU-GM colony numbers of the bone marrow after a single intraperitoneal injection of 5-FU.*

Influence of 5-day oral rosiglitazone pre-treatment in 6 mg/kg dose on bone marrow damage caused by a single 100 mg/kg intraperitoneal dose of 5-fluorouracil (5-FU) indicated by femoral CFU-GM colony numbers. Cultures were grown in triplicates. Values are means ± S.E.M., n=8 in each group, $^{++}$ $P<0.01$, $^{+++}P<0.001$ compared with the values of the control group, *** $P<0.001$ compared to mice treated with 5-FU alone. Mice in control group were treated with vehicle. Group C: control, group R: rosiglitazone-treated (6mg/kg), group 5-FU: 5-FU-treated (100 mg/kg), group 5-FU+R: rosiglitazone (6 mg/kg) + 5-FU-treated

Same effects were also observed conserning the comparison between the bone marrow content of pretreated and non-pretreated mice, allowing only one hour for regeneration after 5-FU injection. Groups receiving pretreatment showed a higher preservation of the CFU-GM pool in bone marrow. Additionally the content of progenitor cells seemed to have some what recoverd in groups receiving pretreatment of rosiglitazone (P<0.001) (Fig 9).

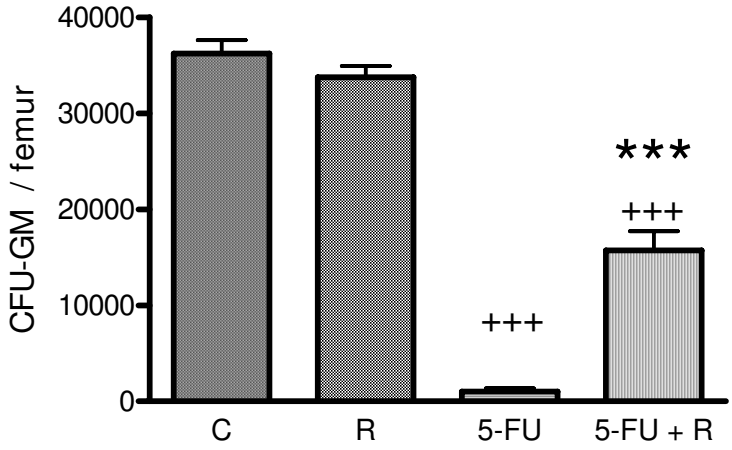

Fig 9. *Effect of rosiglitazon on CFU-GM content of the bone marrow after a single intraperitoneal injection of 5-FU.*

Influence of 5-day oral rosiglitazone pre-treatment in 6 mg/kg dose on bone marrow damage caused by a single 100 mg/kg intraperitoneal dose of 5-fluorouracil (5-FU) indicated by femoral CFU-GM content . Cultures were grown in triplicates. Values are means ± S.E.M., n=8 in each group, $^{++}P<0.01$, $^{+++}P<0.001$ compared with the values of the control group, *** $P<0.001$ compared to mice treated with 5-FU alone. Mice in control group were treated with vehicle. Group C: control, group R: rosiglitazone-treated (6mg/kg), group 5-FU: 5-FU-treated (100 mg/kg), group 5-FU+R: rosiglitazone (6 mg/kg) + 5-FU-treated

4.6. Effects of rosiglitazone pre-treatment on recovery of damaged bone marrow after a single dose of 5-FU.

Characterizing bone marrow function by total cellularity, CFU-GM colony formation and CFU-GM content of femoral bone marrow, damage to the bone marrow function was serious even on the 3rd day after a single dose of 100 mg/kg of 5-FU. Total cellularity and frequency of CFU-GM cells were 30 % and the femoral content of CFU-GM was only 10% of the control value. Pre-treatment with 6 mg/kg rosiglitazone for 5 days resulted in significant improvement of these variables. The total cellularity and the CFU-GM pool increased to 50% of the control values and frequency of CFU-GM cells was normal even on the 3rd day of the

regeneration. Recovery of hemopoiesis was very slow after bone marrow damage caused by 5-FU. By the 6th day cellularity stood at 30 % after a nadir of 18% and CFU-GM content came up to only 40 % of the control value despite the elevated intensive proliferation of the CFU-GM cells (Fig 10).

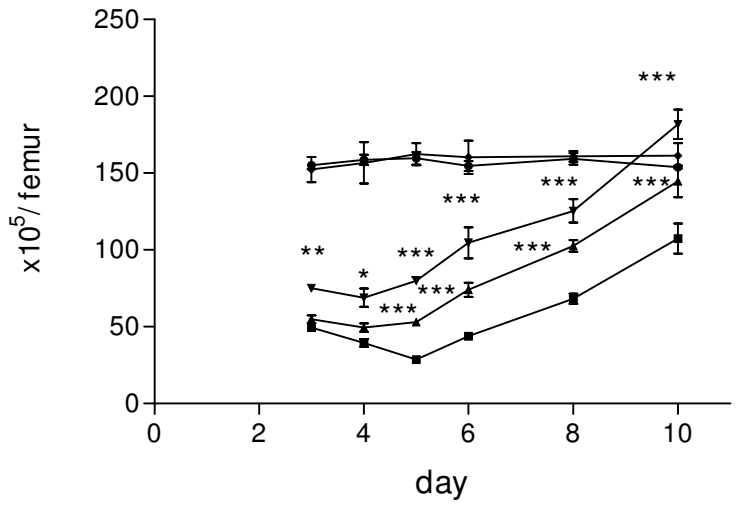

Fig 10. *Rosiglitazon effect on cellularity as a function of time*

Influence of 5-day–long oral rosiglitazone pre-treatment in doses of 3 and 6 mg/kg on recovery of the 5-fluorouracil-damaged bone marrow indicated by total cellularity of the bone marrow. Days are numbered from the single intraperitoneal injection of 5- fluorouracil. Cultures were grown in triplicates. Values are means ± S.E.M., n=13 in each group, **P*<0.05, ** *P*<0.01, *** *P*<0.001 compared to mice treated with 5- fluorouracil alone. Mice in control group were treated with vehicle. (groups see in Fig 12, p 44)

Recovery of bone marrow functions was accelerated by the rosiglitazone pre-treatment. Higher intensity of proliferation in CFU-GM cells resulted in normal colony numbers as early as 4th

day using 3 mg/kg doses and normalization happened on 3rd day with 6 mg/kg doses. The enhanced proliferation of these groups is highlighted when compared to the group treated by 5-FU alone, where standard colony numbers were reached on the 5th day (Figure 11).

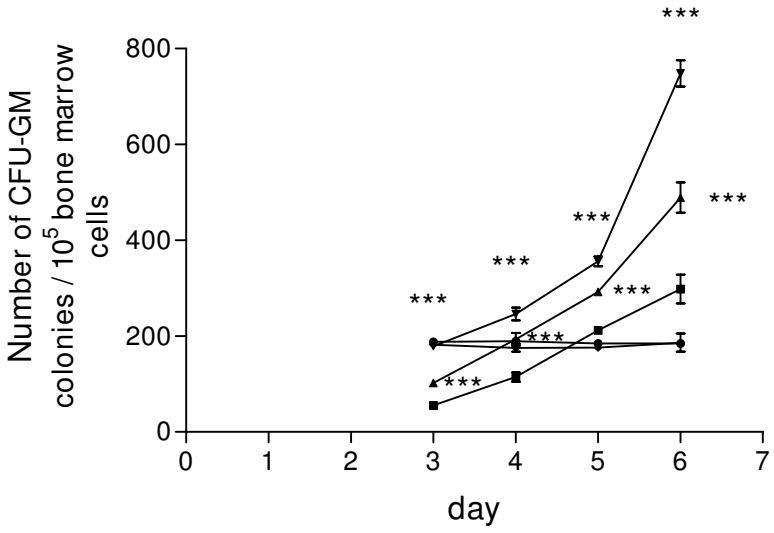

Fig. 11. *Rosiglitazon effect on CFU-GM colony numbers as a function of time*
Influence of 5-day–long oral rosiglitazone pre-treatment in doses of 3 and 6 mg/kg on recovery of the 5-fluorouracil-damaged bone marrow indicated by CFU-GM colony numbers of the bone marrow. Days are numbered from the single intraperitoneal injection of 5-fluorouracil. Cultures were grown in triplicates. Values are means ± S.E.M., n=13 in each group, **P*<0.05, ** *P*<0.01, *** *P*<0.001 compared to mice treated with 5- fluorouracil alone. Mice in control group were treated with vehicle. (groups see in Fig 12, p 44)

CFU-GM pool was replenished earlier in rosiglitazone pre-treated groups and was normalized on the 6th day using 3 mg/kg doses. In addition expansion on 2.5-fold more than normal was observed using 6 mg/kg doses. In the same time frame the group treated with 5-FU alone reached only 40% of the normal CFU-GM pool (Fig 12).

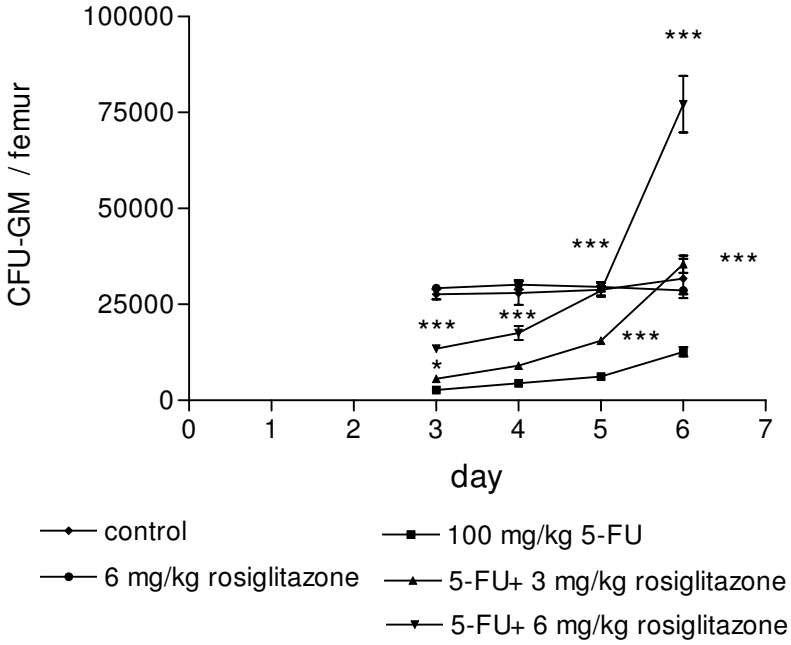

Fig12 *Rosiglitazon effect on CFU-GM content as a function of time*

Influence of 5-day–long oral rosiglitazone pre-treatment in doses of 3 and 6 mg/kg on recovery of the 5-fluorouracil-damaged bone marrow indicated by femoral CFU-GM content . Days are numbered from the single intraperitoneal injection of 5- fluorouracil. Cultures were grown in triplicates. Values are means ± S.E.M., n=13 in each group, **P*<0.05, ** *P*<0.01, *** *P*<0.001 compared to mice treated with 5- fluorouracil alone. Mice in control group were treated with vehicle.

4.7. Effect of rosiglitazone on recovery of white blood cell counts and absolute neutrophil cell counts after a single dose of 5-FU

Numbers of the mature cells originated from the hemopoiesis were decreased after a latent period in peripheral blood, as the previously formed cells were present. White blood cell counts and absolute neutrophil counts decreased from the 4th day (Fig 13).

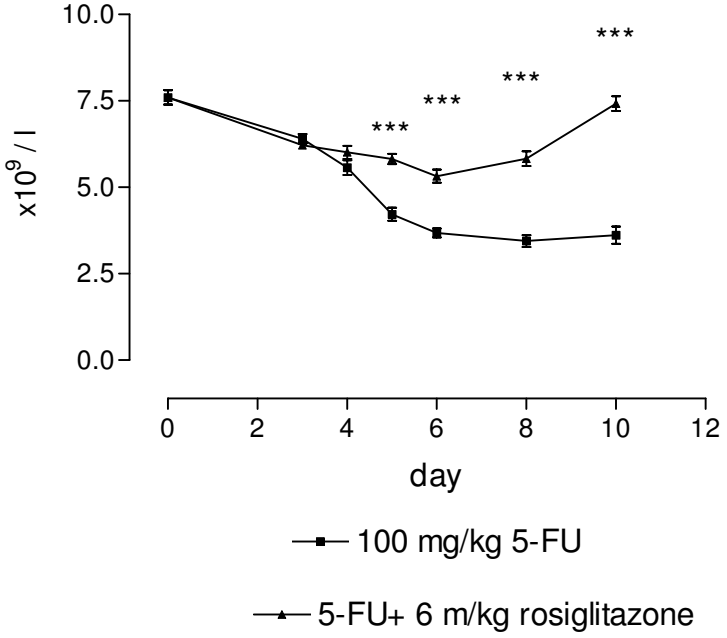

Fig 13 *Effect of rosiglitazone on recovery of periferal blood cell (WBC) after a single 5-FU dose.*

Influence of 5-day oral 6 mg/kg of rosiglitazone pre-treatment on recovery of white blood cell counts in peripheral blood. Days are numbered from the single intraperitoneal injection of 5-fluorouracil. Values are means ± S.E.M., n=13 in each group, **P*<0.05, ** *P*<0.01, ****P*<0.001 compared to mice treated with 5- fluorouracil alone

Especially marked slope in levels of absolute neutrophil counts (ANC) was seen and a slow recovery phase in the remaining days of the observed period. Mice pre-treated with rosiglitazone had a milder decrease in the ANCs and also to a lesser extent (P<0.05-0.001). Until the 10^{th} day after the 5-FU dose, the ANCs remained significantly higher than that of the non- pretreated mice (Fig14).

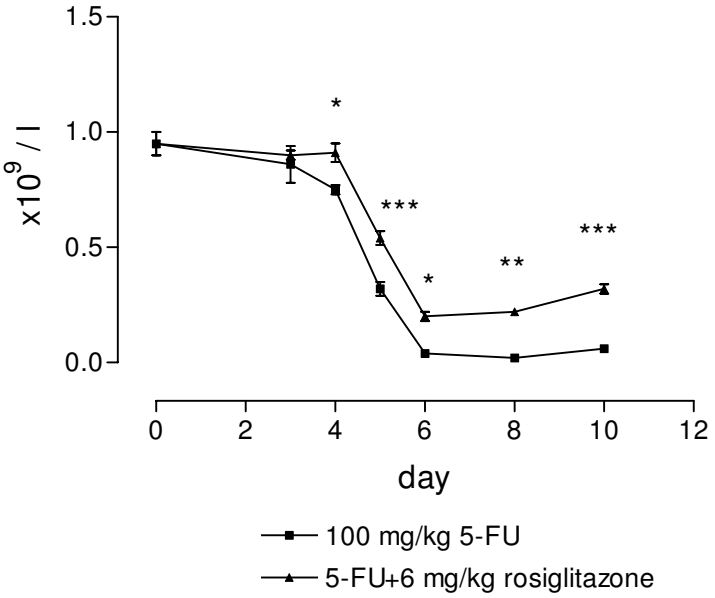

Fig 14. *Effect of rosiglitazone on recovery of absolute neutrophil cell counts (ANC) after a single 5-FU dose.*

Influence of 5-day oral 6 mg/kg of rosiglitazone pre-treatment on recovery of absolute neutrophil counts in peripheral blood. Days are numbered from the single intraperitoneal injection of 5- fluorouracil. Values are means ± S.E.M., n=13 in each group, **P*<0.05, ** *P*<0.01, ****P*<0.001 compared to mice treated with 5- fluorouracil alone.

4.8. Effect of rosiglitazone on granulocyte-macrophage progenitor cells damaged by repeated doses of 5-fluorouracil

Following a 7-day-long daily treatment with a combination of intraperitoneal 5-FU and oral rosiglitazone, bone marrow samples were examined for functional evaluation, having allowed only one hour for regeneration after the last 5-FU injection. Cellularity as well as content of the bone marrow in pretreated mice stood significantly higher than the ones only exposed to 5-FU (Fig. 15).

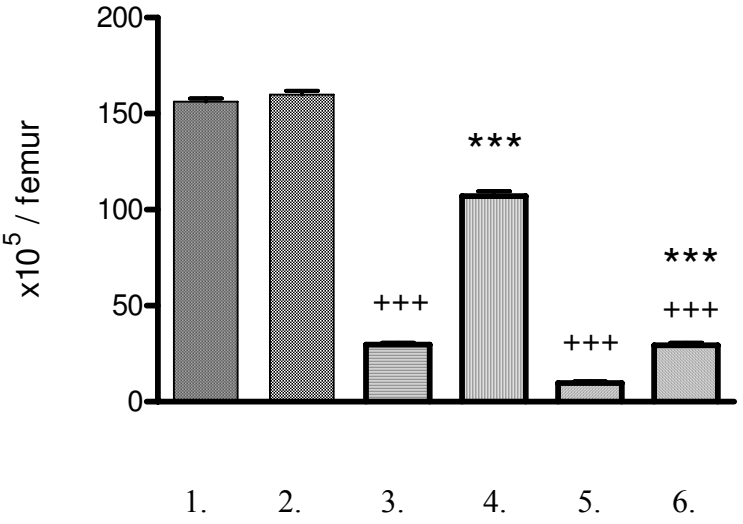

Fig. 15. *Rosiglitazon effect on cellularity after repeated doses of i.p. 5-FU as a function of time.*

Influence of rosiglitazone (6 mg/kg p.o.) administered with intraperitoneal injection of 5-fluorouracil at 25 or 50 mg/kg for 7 days on total cellularity of the bone marrow . Cultures were grown in triplicates. Values are means ± S.E.M., n=8 in each group, $^{+++}P<0.001$ compared with the values of the control group, *** $P<0.001$ compared to mice treated with 5-FU alone. Mice in control group were treated with vehicle. Group 1: control, group 2: rosiglitazone (6 mg/kg)-treated, group 3: 25 mg/kg of 5-FU-treated, group 4: 50 mg/kg of 5-FU-treated, group 5: rosiglitazone + 25 mg/ kg of 5-FU-treated, group 6: rosiglitazone + 50 mg/kg of 5-FU-treated

The more intensive proliferation of CFU-GM cells was indicated by their higher frequencies in femoral bone marrow samples (Fig. 16).

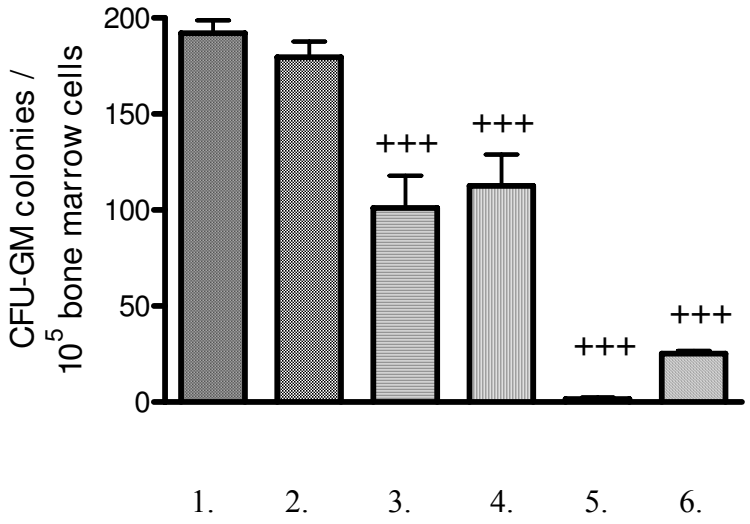

Fig 16. *Rosiglitazon effect on CFU-GM colony numbers of bone marrow after repeated doses of i.p. 5-FU as a function of time*

Influence of rosiglitazone (6 mg/kg p.o.) administered with intraperitoneal injection of 5-fluorouracil at 25 or 50 mg/kg for 7 days on CFU-GM colony numbers of the bone marrow . Cultures were grown in triplicates. Values are means ± S.E.M., n=8 in each group, $^{+++}P<0.001$ compared with the values of the control group, *** $P<0.001$ compared to mice treated with 5-FU alone. Mice in control group were treated with vehicle. . Group 1: control, group 2: rosiglitazone (6 mg/kg)-treated, group 3: 25 mg/kg of 5-FU-treated, group 4: 50 mg/kg of 5-FU-treated, group 5: rosiglitazone + 25 mg/ kg of 5-FU-treated, group 6: rosiglitazone + 50 mg/kg of 5-FU-treated

Similarly the content of the bone marrow in pretreated mice stood significantly higher than the ones only exposed to 5-FU (Fig. 17).

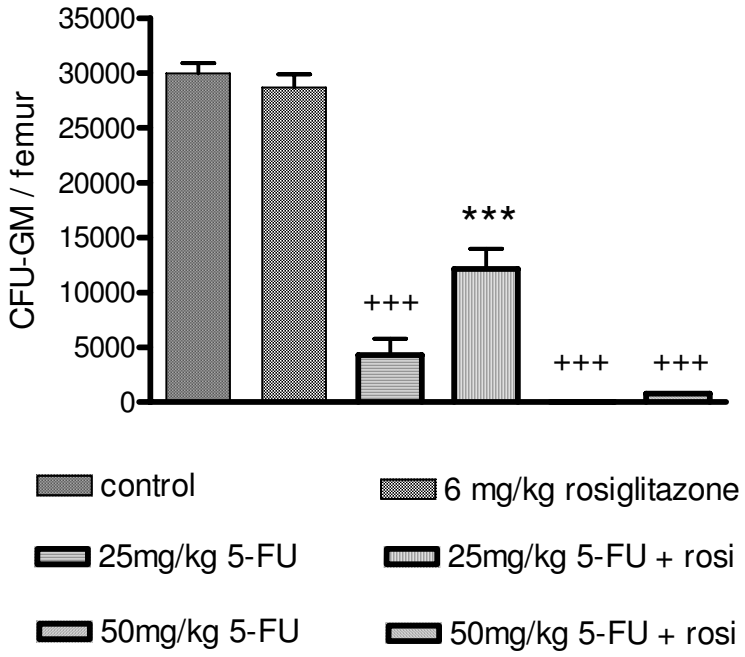

Fig 17. *Rosiglitazon effect on CFU-GM content of bone marrow after repeated doses of i.p. 5-FU as a function of time*

Influence of rosiglitazone (6 mg/kg p.o.) administered with with intraperitoneal injection of 5-fluorouracil at 25 or 50 mg/kg for 7 days on CFU-GM content of bone marrow . Cultures were grown in triplicates. Values are means ± S.E.M., n=8 in each group, $^{+++}P<0.001$ compared with the values of the control group, *** $P<0.001$ compared to mice treated with 5-FU alone. Mice in control group were treated with vehicle.

4.9. Effect of rosiglitazone on blood glucose and insulin levels

Insulin levels tend to increase after a 5-day rosiglitazone treatment. In the group pre-treated with 6 mg/kg of rosiglitazone before the 5-fluorouracil dose, a slightly increased plasma insulin was observed in comparison with the mice treated with 5-fluorouracil alone (Fig.18).

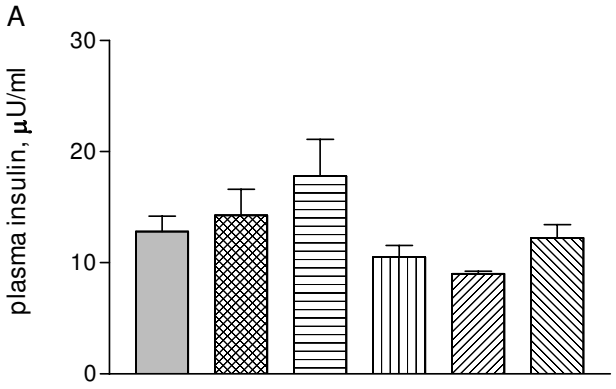

Fig 18. *Effects of rosiglitazone on blood insulin levels.*

Influence of 5-day-long oral rosiglitazone treatment alone and in combination with a single 5-fluorouracil dose on the 5th day on plasma insulin. Cultures were grown in triplicates.Values are means ± S.E.M., n=13 in each group **P*<0.05 compared to the control group treated with vehicle. (groups see in Fig 19)

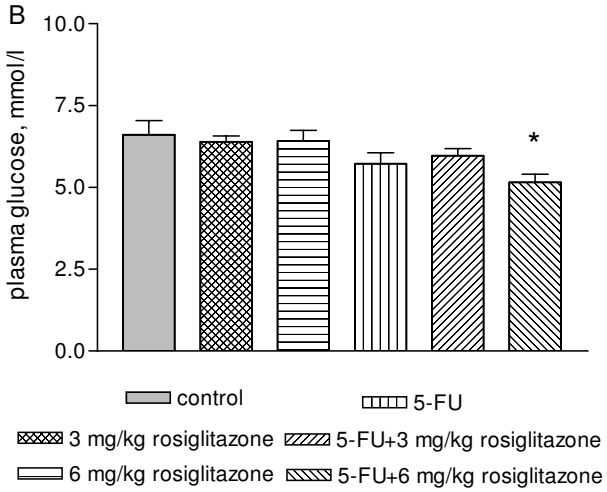

Fig 19. *Effects of rosiglitazone on blood glucose levels.*

Influence of 5-day-long oral rosiglitazone treatment alone and in combination with a single 5-fluorouracil dose on the 5th day on plasma glucose levels measured 2 hours after the 5-fluorouracil i.p. injection . Cultures were grown in triplicates Values are means ± S.E.M., n=13 in each group **P*<0.05 compared to the control group treated with vehicle.

After a 5-day rosiglitazone treatment in groups pre-treated with 6 mg/kg of rosiglitazone before the 5-fluorouracil dose a significant decrease in plasma glucose occured compared with the mice treated with 5-fluorouracil alone.(Fig19)

4.10. Effect of rosiglitazone on damage of murine CFU-GM progenitors *in vitro*

The femoral bone marrow cell suspension was divided into six portions and cells were grown in the presence of rosiglitazone at a concentration of 1 □M. On the 5th day of the culturing period 5-fluorouracil was added to the cultures in a 1 mg/l final concentration. As demonstrated pretreated cultures show a lesser degree of damage and higher number of CFU-GM progenitors preserved.(Fig20)

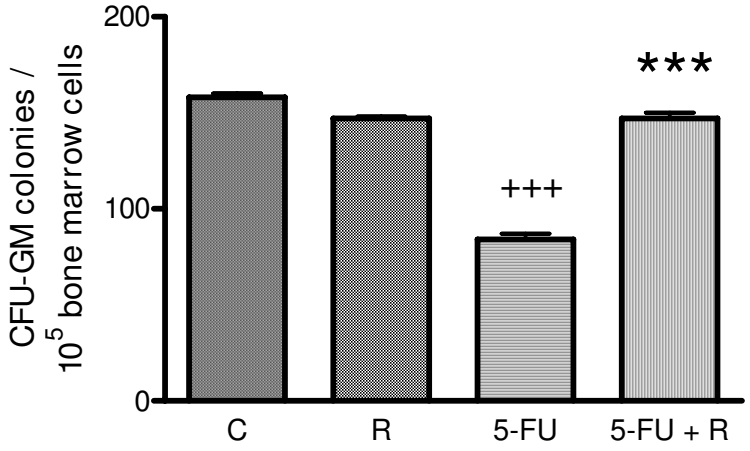

Fig 20. Effect of rosiglitazone on damage of murine CFU-GM progenitors *in vitro*.
Influence of rosiglitazone application at 1 microM concentration in vitro on colony formation of murine CFU-GM progenitor cells cultured in the presence of 5-fluorouracil (1 mg/l) from the 5th day of the cultural period, versus the cultures treated with 5-FU alone in the same manner. Cultures were grown in triplicates. Values are means ± S.E.M., ***P<0.001 compared to cultures treated with 5-FU alone. In control cultures vehicles were used in the same manner. Group C: control, group R: rosiglitazone-treated (6mg/kg), group 5-FU: 5-FU-treated (100 mg/kg), group 5-FU+R: rosiglitazone (6 mg/kg) + 5-FU-treated

4.11. Effects of PPARγ antagonist on rosiglitazone-induced protection *in vitro*

Rosiglitazone is a partial agonist on PPARγ receptors. We investigated whether it has a direct effect on CFU-GM progenitors and whether PPARγ receptor effects are involved in protection. Neither rosiglitazone nor a PPARγ receptor antagonist drug (GW9662) affected colony formation of CFU-GM progenitor cells but they influenced their sensitivity to 5-fluorouracil. Colony formation of CFU-GM progenitors after 5-fluorouracil application was increased if they were grown in the presence of rosiglitazone compared with cells cultured without rosiglitazone. This beneficial effect was neutralized by the presence of the PPARγ antagonist (Fig 21).

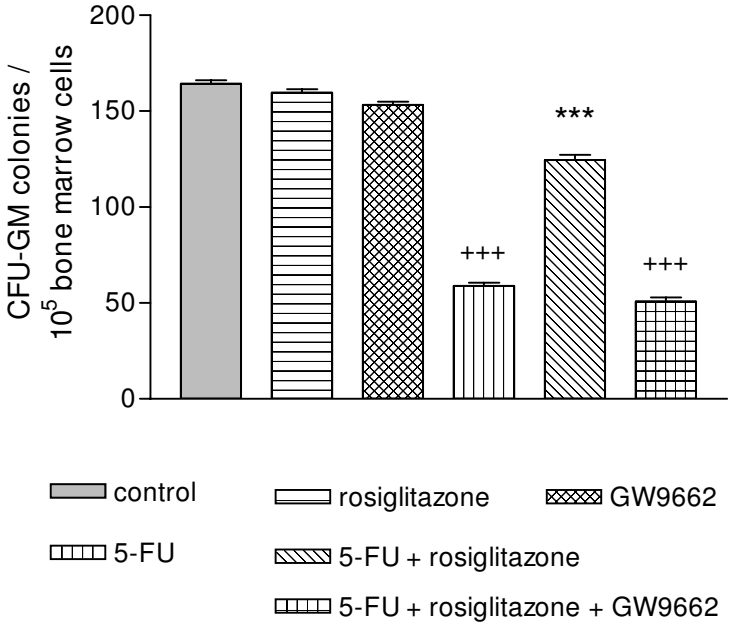

Fig 21.Effects of PPARγ antagonist on rosiglitazone-induced protection *in vitro.*
Influence of rosiglitazone (1μM) and a PPAR-γ antagonist (GW9662, 5 μM) application on damage caused by 5- fluorouracil administered in 1 mg/l final concentration on the 5th day of the cultural period indicated by the colony formation of murine CFU-GM progenitor cells.

4.12. Effects of rosiglitazone on damage of human mobilized peripheral blood stem cells *in vitro*

To detect whether previous beneficial effects of rosiglitazone had a direct or an indirect influence on the hemopoietic cells, in vitro cultures were used. The chosen human mobilized peripheral blood stem cell suspension contained many types of hemopoietic progenitor and stem cells. In our experimental system their differentiation into granulocyte-macrophage cells could be studied, as the grown colonies were especially matured granulocytes and monocytes. Cells were cultured both in presence and absence of rosiglitazone. 5-FU was added to cultures later, on the 5th day. In 1 mg/l concentration 5-FU suppressed colony formation in 87 % of the samples. Rosiglitazone protected progenitor and stem cells dose-dependently against 5-FU damage. Colony formation was inhibited only by 42 % in the presence of 0.5 mM of rosiglitazone. One mM of rosiglitazone enhanced differentiation of stem and progenitor cells towards granulocytes and macrophages to 300% of the control values (Fig 22).

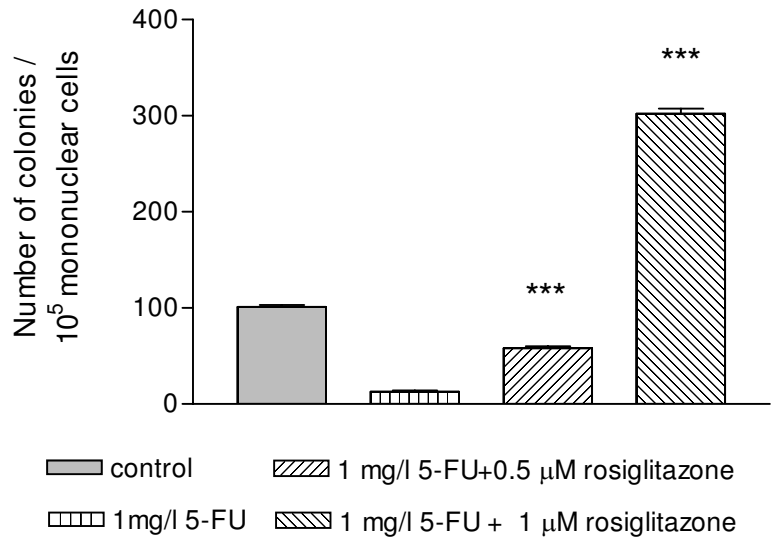

Fig 22. Effects of rosiglitazone on damage of human mobilized peripheral blood stem cells *in vitro*

Influence of rosiglitazone application at concentrations of 0.5 or 1 μM on colony formation of human mobilized peripheral blood stem cells after administration of 5-fluorouracil on the 5th

day of the cultural period in 1 mg/l final concentration, versus the cultures treated with 5-fluorouracil alone in the same manner. Cultures were grown in triplicates. Stem cells were originated from the peripheral blood of three patients. Values are means ± S.E.M., ***P<0.001 compared to cultures treated with 5- fluorouracil alone. In control cultures vehicle was used in the same manner.

5. DISCUSSION

Technical developments and new drugs against malignant cells, as well as the pharmacological modulation of bone marrow toxicity is expected to influence modern chemotherapy for cancer patients. This will hopefully lead to an increase in cure rates with better quality of life. Cancer chemotherapy causes severe damage to hematopoietic stem cells. Myelosuppression is the most common dose-limiting side effect of chemotherapy and can also be the most lethal. Consequently neutropenia is a serious risk for chemotherapy, is associated with infectious complications, necessitating the use of intravenous antibiotics, hospitalization, and may even result in death. The occurrence of febrile neutropenia can lead to dose reductions and delay in subsequent cycles of chemotherapy that may have a detrimental affect on overall survival and disease-free intervals. Therefore introducing means to provide myeloprotective effects is essential, but there are only a few myeloprotective drugs, such as colony stimulating factors (CSFs) and amifostine which are routinely used in clinical practice to reduce acute toxicities of chemo and radiotherapy in cancer patients. Physiological agents are among the first category to be used to increase the differentiation in bone marrow cell lines, starting with colony stimulating factors.

Currently granulocyte-colony stimulating factor (G-CSF) is considered to be the most effective agent among myeloprotective drugs, and used most frequently due to its less side effect profile compared to other hematopoietic growth factors (Esser and Brunner 2003). Filgrastim is a granulocyte colony-stimulating factor (G-CSF) and is also known as recombinant methionyl human granulocyte colony-stimulating factor (r-metHuG-CSF).

Because filgrastim is produced in the bacteria *Escherichia coli*, it is nonglycosylated and is therefore different from G-CSF isolated from human cells. Filgrastim stimulates the proliferation, differentiation commitment, and some end-cell functional activation of hematopoietic cells. Filgrastim is indicated to lessen neutropenia associated with myelosuppressive chemotherapy (including those with acute myeloid leukemia), bone marrow transplantation, and severe chronic neutropenia including those with congenital, idiopathic, and cyclic neutropenia. It is also indicated for the mobilization of peripheral blood progenitor cells (PBPC) prior to PBPC collection. Filgrastim may also be useful in preventing opportunistic diseases in patients infected with the human immunodeficiency virus. Other examples would be molgramostim which is an E. coli-derived, non-glycosylated, recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) used in the treatment of chemotherapy-induced neutropenia. Inconclusive data exist on the use of CSFs with other conditions, especially febrile neutropenia. Although growth factors have made a tremendous impact on decreasing duration of neutropenia thereby decreasing the incidence of infections, reduced efficacy occurs with multiple courses and with bone marrow depletion. Growth factors are proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation. Many growth factors are quite versatile, stimulating cellular division in numerous different cell types; while others are specific to a particular cell-type so not surprisingly another limitation is that there are specific lineage growth factors affecting only a segment of one tissue compartment. Studies continue to find the most effective growth factor. The second category is simple molecules such as amifostine (ETHYOL). This chemical compound has been under investigation for many years, a thiophosphate namely aminothiol cytoprotective agent with the following structural formula: $H_2N(CH_2)_3NH(CH_2)_2S\text{-}PO_3H_2$. Its antioxidant properties may play a role in cytoprotection (Hartmann et al. 2001).

However both filgrastim and amifostine (ETHYOL) are administered parenterally as intravenous or subcutaneous infusions and have been associated with side effects in a large percentage of patients. In case of G-CSF a wide range of symptoms were observed. Symptoms included hypotention, hypoxemia, ascitis, pericardial and pleural effusion, shock, edema, neurological changes, and hepatocellular injury. Most importantly myelodysplastic syndrome with high mortality and capillary leak syndrome were among the reported symptoms. It has been suggested that leukapheresis in a patient with marked leukocytosis

receiving high doses of G-CSF may have triggered neutrophil activation and the release of inflammatory mediators, which resulted in widespread tissue injury and capillary leak syndrome. Also high level of headache, bone/backache, myalgia/arthralgia,need for analgesics, refusal of further therapy, itching insomnia,fatigue/weakness,depression,malaise and to a lesser extent symptoms such as thirst and nausea was experienced (Heuft et al., 2004). Also it should be noted that effect of the most CSFs are not specified to one cell line and since they influence the early pleuripotent cells their use is associated with many side effects such as capillary leak syndrome and eosinophilia.

In case of amifostine mainly nausea (47% gradeI,13% grade II) and hypotention (grade I) and 7% (grade II) was experienced. Apparent nausea and vomiting are the two most prominent side effects of chemo and radiotherapy. If it is aggravated by even the supportive therapy, it will increase the otherwise high percentage of the refusal of the anticancer therapy itself, subsequently having an additive unwanted effects. Therefore it is crucial that the following myeloprotective agents during the chemotherapy treatment be as symptom-free as possible.

The major finding of the present Thesis is that rosiglitazone is able to confer protection on bone marrow cells against 5-FU-induced myelotoxicity. Our finding that a simple thiazolidinedione molecule may have a myeloprotective effect would be an importance in practice. Easily orally administration covers patient's desire and may improve complience. Thiazolidinediones, rosiglitazone and pioglitazone, belong to the most frequently sold 100 pharmaceuticals in the world (Mosby 2003). Of course they have side effects, especially hepatic injury has to be controlled, but millions of people use them without remarkable adverse effects.

5.1. In vivo effects of insulin on granulopoiesis in mice with bone marrow damaged by a cytostatic agent, compared to same effects in healthy bone marrow.

Our hypothesis was to utilize an early-growth factor like effect of insulin. Although insulin is widely used to support colony formation in cultures of hematopoietic progenitor cells, its

effects regarding hematopoiesis *in vivo* have not been documented. Our aim was to study whether insulin itself could influence myelopoiesis *in vivo* in mice.

Insulin had no effects on colony formation of CFU-GM in healthy bone marrow. This may be one of the reason that in vivo experiments have been neglected. But it could preserve more progenitor cells in damaged marrow. Hematopoiesis is controlled by a network of cytokine interactions. Many parallel and combined effects converge in hematopoiesis. It is not surprising that neither insulin nor rosiglitazone were able to enhance the optimal colony numbers in balanced normal hematopoiesis in our experiments. But in damaged hematopoiesis they were able to help restore the normal size and ratio of the cell compartments in bone marrow more quickly. Insulin itself has no stimulative effect on progenitor cells *in vitro*, but it can increase their survival (Ratajczak et al., 1998). Equilibrium between survival and apoptosis can be shifted towards survival by insulin (Iida et al., 2002). In the very flexible hematopoietic system some growth factors are able to substitute for each other and many of them can potentiate each other's effects by increasing sensitivity to the subsequent factor during differentiation. By increasing survival and the amplifying the effects of the other colony stimulating factors insulin or rosiglitazone can enlarge the damaged CFU-GM pool and accelerate bone marrow regeneration. Insulin showed a stronger protective effect at lower doses of 5-fluorouracil. A more serious degree of damage could moderate this protection, as was seen in our experiments.

5.2. An insulin sensitizer drug, namely rosiglitazone has similar effects on normal and damaged bone marrow than insulin

After determining insulin sensitizing dose range, similar doses were used in experiments to evaluate whether rosiglitazone has myeloprotective effects. Since insulin has many metabolic effects and can easily produce hypoglycemia it is much more convenient to use an agent with same property and more moderate effect such as insulin sensitizers e.g. rosiglitazone. Since in vivo bone marrow protection is more advantageous, we also tested if rosiglitazone, a thiazolidinedione, could prevent anticancer drug induced myelotoxicity by increasing the host's insulin sensitivity.

Rosiglitazone similar to insulin has also no effect on colony formation of CFU-GM progenitors in healthy bone marrow, but administered in similar pattern before 5-FU-caused damage it is able to confer protection on bone marrow cells against 5-FU-induced toxicity. Two days after the single high 5-FU dose significantly more CFU-GM cells were observed in femoral bone marrow. The increase of the frequency of CFU-GM progenitors without change in bone marrow cellularity in the rosiglitazone pre-treated murine groups following 5-FU indicates intensive proliferation of these progenitor cells. The expansion of the CFU-GM pool is required to compensate for the 5-FU-induced loss and allows faster recovery. Granulocyte colony-stimulating factor, which is recently used for facilitating recovery from neutropenia, increases CFU-GM content to twofold in the 5-FU-damaged bone marrow in mice (Gilmore et al., 1995). Rosiglitazone pre-treatment could enhance the CFU-GM content to 3.7fold compared to that in mice treated by 5-FU alone.

We could also evaluate that even immediately after the 5-FU-caused damage were significantly more progenitor cell survived in the pre-treated mice than their 5-FU-treated counterparts. It means that the expansion of CFU-GM compartment based upon at least in part a decreased susceptibility of granulocyte-macrophage progenitors to 5-FU-toxicity *in vivo* by the end of the 5-day rosiglitazone pre-treatment. Rosiglitazone could preserve more progenitor cells for recovery.

5.3. How can rosiglitazone influence the recovery of bone marrow as a function of time?

Rosiglitazone, a thiazolidinedione insulin sensitizer drug, was investigated in mice as a possible protector against 5-fluorouracil-induced myelotoxicity. Such protective effects offer significant clinical application only if a considerable enhancement in restoration of stem cells and facilitation of the recovery phase after injury can be demonstrated. We could state that the non-pre-treated groups reached their intensified regeneration phase following bone marrow damage induced by a cytostatic agent at a later time than groups pre-treated with rosiglitazone. Significant dose-dependent differences were demonstrated by comparing the degree of bone marrow regeneration following 5-fluorouracil injections, as indicated by CFU-GM content and frequency. The high intensity of proliferation in turn resulted in earlier

recovery of the CFU-GM pool. Therefore it was logical to conclude that healing started earlier in groups receiving rosiglitazone.

Rosiglitazone is not unique in affecting colony formation of some progenitor cells, i.e. granulocyte-macrophage progenitors. It was shown that rosiglitazone could increase colony formation of bone marrow-derived primitive progenitor cells and promote their differentiation towards the endothelial lineage in mice (Wang et al., 2004). The effective dose range was similar to that which we used in our experiments.

5.4. Whether is rosiglitazone able to mitigate the severity of neutropenia during the recovery of bone marrow after cytostatic drug-induced damage ?

The same protective effect was reflected by the absolute neutrophil counts in peripheral circulation. The nadir of absolute neutrophil count was less deep in pre-treated mice and remained significantly higher during the observed period.

The myeloprotective property of rosiglitazone may be due to a range of direct and indirect effects. Some insulin-like effects could be participating in the observed myeloprotection, since the dose range, which increased insulin sensitivity, was similar to that resulting in increased CFU-GM colony numbers. Additionally we have found the expansion of CFU-GM pool a characteristic effect of insulin, in rosiglitazone pre-treated mice. The subcutaneous insulin used in similar treatment protocols also increased the frequency and femoral content of CFU-GM in 5-fluorouracil-damaged bone marrow in mice. *Ex vivo* insulin is added to the culture media when growing and expanding human CD34+ cells (Mobest et al., 1998). Insulin, if added alone to human CD34+ cells cultured under serum free conditions, does not stimulate their proliferation, rather it augments the viability of haematopoietic progenitors by preventing them from undergoing apoptosis (Ratajczak et al., 1998). Rosiglitazone ’s effects are similar to those of insulin on haematopoietic progenitors. According to Ratajczak et al. insulin rather potentiate stimulatory effects of other growth factors on proliferation of bone marrow progenitors, and it augments their viability in serum-free in vitro cultures. Rosiglitazone, similarly to insulin, had no effect on CFU-GM progenitor cell compartment in

mice *in vivo,* but could protect it against 5-FU toxicity. Based on these results we feel inclined to believe that rosiglitazone at least in part acts to amplify the effects of insulin on the progenitor cells. Insulin-receptor substrate protein IRS-1 mediates stimulatory effects of insulin in haematopoietic cells shared among some other growth factors e.g. insulin-like growth factor-I (IGF-I) in regulating cell growth and metabolism (Wang et al. , 1993,Uddin et al., 1997). Rosiglitazone decreases inhibitory serine phosphorylation of IRS1 *in vitro* and *in vivo* (Jiang et al., 2004). Although the effects of insulin on myelopoiesis have not been investigated *in vivo*, the stimulating effects of insulin-like growth factor-I have been observed on myelopoiesis in mice treated with azidothimidine (Tsarfaty et al., 1994, Difalco et al., 1998).

5.5. Whether is rosiglitazone able to protect granulocyte-macrophage progenitor cells in bone marrow damaged by repeated doses of 5-fluorouracil?

In clinical practice cytostatic drugs are administered over short intensive periods, simultaneous administration of myeloprotective agents would be desirable. To fit protective agents to the treatment schedules of malignant diseases is not easy. Haematopoietic colony-stimulating factors are used frequently to moderate myelotoxicity, but administration of granulocyte colony-stimulating factor G-CSF, the recently used agent supporting bone marrow regeneration, prior to chemotherapy actually may worsen the toxic effects on bone marrow (De Wit et al., 1996). G-CSF has significant beneficial effects only if it is administered after chemotherapy (Timmer-Bonte et al., 2005). This is important in the design of clinical cancer treatment protocols. We expanded our studies to determine whether concurrent use of 5-FU in multiple doses with rosiglitazone influences the observed myeloprotection. Kinetics of progenitor cell populations when chemotherapeutic agents are administered seemed to play an important role in neutropenia after chemotherapy. This should be taken into consideration when administering a stimulatory haematopoietic growth factor, to counter effects of chemotherapy (De Wit et al., 1996 , Misaki et al. 1998) found that 2-day G-CSF free interval before the single cytostatic dose decreased myelotoxicity while additional pre- and post-treatment with G-CSF even aggravated it.

In contrast to these observations concurrent use of rosiglitazone with 5-FU repeatedly during 7 consecutive days did not unfavourably influence the population of the CFU-GM progenitor

cells. The increased femoral content of CFU-GM immediately after the single high dose of 5-FU showed that rosiglitazone pretreatment preserved more progenitors for the recovery of bone marrow. The increased frequency of CFU-GM cells with their intensive proliferation allowed faster restoration of the damaged CFU-GM compartment than was seen even during repeated administration of the cytostatic drug.

One possible explanation is that rosiglitazone itself had no stimulatory effect on progenitor cells even in repeated doses compared with the effects of G-CSF. G-CSF markedly increases both numbers of CFU-GM progenitors and absolute neutrophil counts in healthy volunteers and animals (Goebel et al., 2004, Hernandez-Bernal et al., 2005). Rosiglitazone 's effects are similar to those of insulin on haematopoietic progenitors and at least in part it acts to amplify the effects of insulin on the progenitor cells with synergizing stimulatory effects of other growth factors on proliferation of bone marrow progenitors, and increasing their viability.

Rosiglitazone pre-treatment could preserve 15 times more CFU-GM progenitor cells compared to that in mice treated by 5-FU alone and even in the case of simultaneous administration with repeated doses of 5-FU, the expansion of the CFU-GM compartment was 3 times and 50 times greater in the combined-treated mice than in mice treated with 25 and 50 mg kg-1 repeated doses of 5-FU, respectively.

Although CFU-GM compartment is expanded significantly in combined treated groups when compared with their counterparts treated by repeated 5–FU doses alone, differences in absolute neutrophil counts were not significant. During 5-FU treatment the more primitive stem cells enter cycle and in the proliferative phase they are more sensitive to effects of 5-FU. The stem cell compartments diminish in bone marrow using repeated doses of 5-FU (Goebel et al. 2004). As Harrison and Lerner (1991) observed the most serious damage if the second dose (150 mg/kg) was administered on 3^{rd} or 5^{th} days to the mice. In our experiments less daily doses of 5-FU were used for 7 days. By the 7^{th} day also the cellularity of the bone marrow decreased as a result of the supply of granulocytes and macrophages breaking away from the previously formed matured cells in the bone marrow and into the blood. The normal haematopoietic progenitor cells were damaged to a greater extent by the repeated 50 mg/kg doses of 5-FU than the single 100 mg/kg dose. Both the frequency and the content of CFU-GM of the femoral bone marrow were significantly less ($P<0.05$). The neutrophil granulocytes disappeared from the blood in majority of cases and despite expansion of CFU-

GM progenitor compartment due to rosiglitazone treatment, more time is required for their maturation to granulocytes before the next 5-FU dose. Rosiglitazone with these daily doses might improve neutropenia if longer intervals or shorter cycles are used in administration schedule of 5-FU.

5.6. Whether has rosiglitazone any effect on plasma insulin and glucose levels?

Although hyperinsulinemia is not expected with rosiglitazone treatment (Zawalich et al., 2003, Seda et al., 2002), a tendency toward increased plasma insulin levels was observed in groups treated with 6 mg/kg of rosiglitazone. The decrease in plasma glucose level was significant in mice treated with 6 mg/kg of rosiglitazone and 5-fluorouracil in combination, but it remained within normal range. Some authors published that rosiglitazone can improve insulin secretory responses of pancreatic beta cells to oscillations in plasma glucose levels (Walter and Lubben, 2005). The effect of this small-scale release of insulin on hematopoietic cells cannot be excluded but the more significant myeloprotection suggests the possibility of other influences. Rosiglitazone may act on progenitor cells indirectly through insulin release or directly by increasing the insulin sensitivity of these cells. A number of other indirect and direct effects could also alter hematopoiesis. Rosiglitazone influences the production of adipocyte-derived factors. These changes may also increase the insulin sensitivity of cells, but some adipokines may also directly regulate myelopoiesis (Bennett et al., 1996).

5.7. Whether has rosiglitazone any direct effects on progenitor cells of the bone marrow in vitro and are there any connection with its PPARγ receptorial effects.?

The myeloprotective property of rosiglitazone may be due to a range of direct and indirect effects. To answer the question whether direct effects on progenitor cells are involved, we studied in vitro cultures of these cells. Similar effects were observed in the in vitro bone marrow cell cultures than *in vivo*. Murine CFU-GM progenitor cells growing in the presence of rosiglitazone were less damaged by 5-FU. Therefore we concluded that the effects of rosiglitazone on bone marrow progenitor cells were direct.

Beneficial effects of thiazolidinediones on cellular glucose metabolism used in the treatment of Type 2 diabetes mellitus (Vamecq and Lattrufe, 1999, El-Batran et al., 2005)] are based on their PPARγ (peroxisome- proliferator-activated receptor gamma) receptorial effects and their influencing the insulin signaling pathway (Uddin et al., 1997, Kramer et al.,2001, Miyazaki et al., 2003). The PPARγnuclear receptor is involved in controlling of proliferation, differentiation and cell death since it often forms heterodimers with retinoid X receptor (RXR) (Frohlich et al., 2005). The role of retinoid receptors in the differentiation of myelopoietic cell lines on which the use of all trans retinoic acid (ATRA) bases in the treatment of acute promyelocytic leukaemia in clinical practice is well-known (Sirulnik et al.,2005). PPARγ receptors are found in many haematopoietic cell lines.
(Greene et al., 2000) and its role has been investigated. We found that a PPARγ antagonist (GW9662) could shield the protective effect of rosiglitazone against 5-FU toxicity to CFU-GM colony formation *in vitro*.

5.8. Whether has rosiglitazone any direct effects on human stem and progenitor cells originated from peripheral blood of patients after their mobilization from bone marrow before autolougous peripheral blood stem cell transplantation?

Autologous peripheral blood stem cell transplantation is now routinely used for patients with certain hematologic malignancies. After mobilization, blood is enriched with a variety of hematopoietic stem and progenitor cells, which can be obtained by leukapheresis. The damage caused by 5-fluorouracil decreased in a dose-dependent fashion in cultures containing rosiglitazone. Rosiglitazone ‘s beneficial modifying effect was observed in the similar dose range on the human progenitor cells, than on the murine CFU-GM., which raises hope for the future use in practice. *In vitro* amifostine increased CFU-GM colony formation from primitive progenitor cells 2-fold (List et al., 1998), and G-CSF in synergism with GM-CSF, SCF, interleukin-3 (IL-3) and IL-6 increased colony formation from cord blood cells 49-fold, (Denning-Kendall et al., 1998). In our experiments rosiglitazone was able to enhance CFU-GM colony formation up to 25-fold in the 5-fluorouracil-damaged human mobilized primitive progenitor cells.

Summarizing our new results:

1. Although insulin is widely used to support colony formation in cultures of hematopoietic progenitor cells, its effects regarding hematopoiesis *in vivo* have not been documented. Insulin had no effects on colony formation of CFU-GM in healthy bone marrow, but it can preserv more progenitor cells in damaged marrow.

2. An insulin sensitizer drug, namely rosiglitazone in insulin-sensitizing dose had similar effects on normal and damaged bone marrow than insulin.

3. Rosiglitazone pre-treatment accelerated recovery of 5-fluorouracil-damaged bone marrow. Consequently neutropenia was milder.

4. G-CSF, the recently used agent supporting bone marrow regeneration, prior to chemotherapy actually may worsen the toxic effects on bone marrow. In contrast to these observations concurrent use of rosiglitazone with 5-FU repeatedly during 7 consecutive days did not unfavourably influence the population of the CFU-GM progenitor cells.

5. The accelerated recovery based upon a decreased susceptibility of CFU-GM progenitors to 5-FU-toxicity by the end of the 5-day rosiglitazone pre-treatment.

6. Myeloprotection was partly due to direct effects on progenitors, regarding similar effects were observed in the in vitro bone marrow cell cultures than *in vivo*.

7. In vitro rosiglitazone's beneficial effects were neutralized by a peroxisome-proliferator-activated receptor gamma (PPARγ) antagonist.

8. Rosiglitazone 's beneficial modifying effect was observed in the similar dose range on the human progenitor cells, than on the murine CFU-GM.

In conclusion

rosiglitazone may have many direct and indirect supportive effects on haematopoiesis, which may be important, especially considering that thiazolidinediones can inhibit many types of malignant cell lines including acute myeloid leukemic cells (Asou et al. 1999, Ohta et al., 2001, Fujimura et al., 1998; Mossner et al., 2002; Toyoda et al., 2002). Rosiglitazone may protect normal CFU-GM progenitor cells against toxicity of cytostatic agents. The myeloprotective effects of rosiglitazone may also prevent infections associated with neutropenia caused by chemotherapy, which is the highest cause of mortality in malignant

diseases. Being able to avoid such disturbing side effects of chemotherapy as oral candidiasis and other chronic mycoses obviously helps to improve quality of life of the patients. It may also have an importance in pharmacological *ex vivo* purging of peripheral blood progenitor cell collections. Pharmacological *ex vivo* purging has been developed to eliminate tumor cells in the autograft using cytostatic drugs, but preserving the normal progenitor cells has proven difficult.

Regarding that the presently used protective agents, haematopoietic stimulating factors and amifostine have uncomfortable side effects in large percentage of patients and they are administered parenterally subcutaneously or intravenously, the easy orally use of thiazolidinediones may be an advantage to improve the complience of the patients. During chemo- and radiotherapy the least tolerable side effects for the patients are nausea and vomiting, which can even lead to refusal of therapy. Aggravating such effects would not be desirable. Amifostine results in nausea and vomiting in about the half of the patients. Rosiglitazone precipitates nausea less than the 0.5 percent of the patients.

Thus rosiglitazone may be a real alternative to the existing myeloprotective drugs in the future due to its easy oral administration and relatively low risk, providing higher life expectancy accompanied by better quality of life for patients with malignant diseases but further studies are warranted to evaluate the optimal treatment schedules.

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8. Publications on which this Thesis is based on:

Original research articles:

1. Benkő, I., K. Djazayeri, C. Ábrahám, J. Zsuga, Z. Szilvássy: Rosiglitazone-induced protection against myelotoxicity produced by 5-fluorouracil.
Eur. J. Pharmacol. 477, 179-182, 2003.

IF: 2,352

2. Djazayeri, K., Z. Szilvássy, B. Peitl, J. Németh, L. Nagy, A. Kiss, B. Szabó, I. Benkő. Accelerated recovery of 5-fluorouracil-damaged bone marrow after rosiglitazone treatment. Eur J Pharmacol 522, 122-129, 2005.

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Other publications:

Abstracts

1. I Benkő, K Djazayeri, P Literati-Nagy, G Rablóczky, J Zsuga, B Szabó, Z Szilvássy: Insulin-sensitization as a novel chemoprotective mechanism.
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Presentations

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2. K. Djazayeri, Z. Szilvássy, I. Benkő: Rosiglitazone, an insulin sensitizing drug, fastened regeneration of bone marrow damaged by 5-fluorouracil in mice. 4th Meeting of the Federation of European Pharmacological Societies, EPHAR, 2004, Porto, Portugal

3. Benkő, I., K. Djazayeri , B. Rózsa, Zs. Kovács, Z. Dinya, A.J. Szentmiklósi: Protective effects of fruit extract with high polyphenol content against doxorubicin-induced myelotoxicity in vivo. 4th Meeting of the Federation of European Pharmacological Societies, EPHAR, 2004, Porto, Portugal

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