Anti-Cancer Drugs 2000, **11,** pp. 79–84

Preclinical report

The effects of granulocyte colony stimulating factor on chemiluminescence and lipid peroxidation of blood platelets treated with cisplatin

Beata Olas,[1] Barbara Wachowicz[1] and Andrzej Buczyński[2]
[1]Department of General Biochemistry, Institute of Biochemistry, University of Łódź, 90-237 Łódź, Poland.
[2]Department of Preventive Medicine, Military School of Medicine, 90-647 Łódź, Poland.

The effects of granulocyte colony stimulation factor (G-CSF) at concentrations of 0.08, 0.8 and 8 μg/ml on reactive oxygen species (ROS) generation and lipid peroxidation induced by cisplatin in pig blood platelets were investigated. The level of reactive oxygen species ($O_2^{\cdot -}$, H_2O_2, singlet oxygen and organic radicals) generated in platelets was measured by the chemiluminescence method. Lipid peroxidation was determined by the thiobarbituric acid technique and was expressed as thiobarbituric acid reactive substances. G-CSF at the concentration of 0.08 μg/ml had a strong inhibitory effect (about 60% inhibition) on the production of ROS in the isolated pig platelets. This cytokine also significantly reduced lipid peroxidation in control platelets and platelets treated with cisplatin ($p<0.05$). In the presence of G-CSF in the incubation medium (0.8 μg/ml) cisplatin-induced generation of ROS was also reduced ($p<0.05$). This study demonstrates that G-CSF has a protective effect against the oxidative stress in blood platelets caused by cisplatin. [© 2000 Lippincott Williams & Wilkins.]

Key words: Blood platelets, chemiluminescence, cisplatin, granulocyte colony stimulating factor, lipid peroxidation, oxidative stress.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum II) is an important chemotherapeutic agent; however, the clinical applications of cisplatin are restricted by severe side-effects including hematological toxicity.[1,2]

Cisplatin can affect blood platelet function *in vitro*. It has an inhibitory effect on platelet activation.[3–6] The detailed molecular mechanism by which this drug can interfere with blood platelet function is not entirely understood, though it has been established that cisplatin induces oxidative stress.[7,8] We showed that cisplatin caused platelet lipid peroxidation concomitant with the inhibition of the antioxidative enzyme system in these cells.[7,9,10] This drug also inhibited the enzymatic peroxidation of platelet endogenous arachidonate.[9,10] Our previous results also revealed that granulocyte colony stimulating factor (G-CSF) reduced the inhibitory action of cisplatin on the thrombin-induced arachidonate pathway in platelets.[11]

G-CSF is a glycoprotein which regulates the production and release of functional neutrophils from the bone marrow. Chemotherapy with administration of G-CSF induced mobilization of peripheral blood progenitor cells, and accelerated both neutrophil and platelet recovery after high-dose chemotherapy.[12–16] The studies *in vitro* and *in vivo* have indicated that combination with cytokines before or during anticancer drug treatment can reduce the toxic effect of cytostatic drugs.[17–19] Some cytokines also have anti-tumor properties against a variety of cancers.[20–23] We studied the effects of G-CSF alone and in combination with cisplatin on the generation of reactive oxygen species (ROS) ($O_2^{\cdot -}$, H_2O_2, singlet oxygen and organic radicals) and lipid peroxidation in isolated pig blood platelets.

Materials and methods

Chemicals

Cisplatin, thiobarbituric acid and luminol were purchased from Sigma (St Louis, MO). G-CSF was obtained from Roche (France) as Neupogen. Stock solution (3000 ng/ml) was stored at 4°C. The final concentrations of G-CSF used were 0.08, 0.8 and 8 μg/ml of platelet suspension. All other chemicals were of AR grade from POCh (Gliwice, Poland).

Correspondence to B Olas, Department of General Biochemistry, Institute of Biochemistry, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland.
Tel/Fax: (+48) 42 6354484;
E-mail: olasb@biol.uni.lodz.pl

Blood platelet preparation

Pig blood was put into ACD solution (citric acid/citrate/dextrose) 5:1 v/v. Platelets were isolated by differential centrifugation of the blood. Blood was centrifuged for 20 min at 200 *g* and the platelet-rich plasma was then centrifuged for 20 min at 1000 *g* to sediment platelets. The resulting pellet was washed twice by centrifugation in modified Ca^{2+}-free Tyrode's buffer (containing 154 mM NaCl, 10 mM Tris–HCl and 5 mM glucose, pH 7.4) and was gently suspended in the same buffer. The platelet suspensions (5 mg of platelet protein/ml) were incubated (5–30 min at 37°C) with (i) G-CSF alone at final concentrations of 0.08, 0.8 and 8 μg/ml, (ii) cisplatin at the final concentration of 20 μM, and G-CSF plus cisplatin.

Lipid peroxidation and ROS generation in blood platelets

Lipid peroxidation. The process of lipid peroxidation in control platelet suspensions and platelets after treatment with G-CSF, cisplatin or G-CSF plus cisplatin was determined by the thiobarbituric acid technique[24] and was expressed as thiobarbituric acid reactive substances (TBARS). After incubation of platelets with the tested drugs, samples of platelets were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged at 12 000 *g* for 15 min. One volume of clear supernatant was mixed with 0.2 volumes of 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured and results were expressed as nmol of TBARS.[24]

Chemiluminescence measurements. The level of ROS ($O_2^{\cdot -}$, H_2O_2, singlet oxygen and organic radicals) in control blood platelets and platelets incubated with G-CSF, cisplatin or G-CSF plus cisplatin was recorded using the chemiluminescence method as described by Król *et al.*[25] The chemiluminescence signals were evaluated by means of a Berthold LB950 automatic luminescence analyzer after the addition of 20 μl of 2 mM luminol solution in buffered saline. Results were expressed as the integral over the total measuring time (per 15 min) and presented as percentage of control values obtained for control platelets.

Platelet protein

Platelet protein was determined by the modified Lowry method.[26]

Statistical analysis

Statistical analysis was done using the Student's *t*-test for paired data.

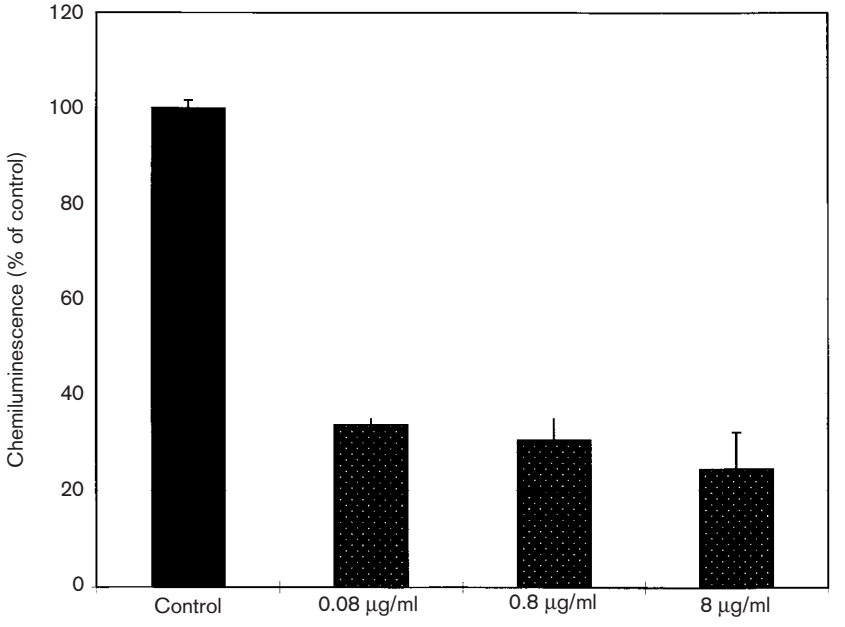

Figure 1. The effect of G-CSF at concentrations of 0.08, 0.8 and 8 μg/ml (5 min, 37°C) on the ROS level in pig blood platelets measured by the chemiluminescence method. Results, expressed as percent of control, are means ± SD of four experiments, $p < 0.05$ (compared with the control).

Results

Our study demonstrates that G-CSF had a strong inhibitory effect (about 60% inhibition) on the generation of ROS in platelets measured by the chemiluminescence method (Figure 1). The inhibitory action of G-CSF on the level of ROS in platelets was dose dependent ($p<0.05$, $R^2=0.9658$, $n=4$) (Figures 1 and 2). The addition of cisplatin (20 μM) to pig platelets caused rapid generation of ROS ($n=4$) (Figure 3). After the incubation of platelets with 20 μM cisplatin together with 0.8 μg/ml G-CSF (5 min, 37°C), the generation of ROS was decreased about 45% ($p<0.05$, $n=4$) (Figure 3). The presence of G-CSF in incubation medium also significantly reduced generation of ROS in control blood platelets (Figure 3).

In another set of experiments we measured the lipid peroxidation in control blood platelets (spontaneous activation) and in platelet suspensions incubated with G-CSF and cisplatin.

Treatment of pig blood platelet suspensions with G-CSF reduced the level of TBARS in a dose-dependent manner (Figures 2 and 4). The inhibitory effect on TBARS production was observed after 5 min action of G-CSF and then slightly increased due to an increase of G-CSF concentration (Figure 2). Cisplatin (20 μM) caused platelet lipid peroxidation ($p<0.01$) (Figure 5) and incubation of these cells with G-CSF (30 min) at a concentration of 0.8 μg/ml had an inhibitory effect on TBARS production (Figure 5).

Discussion

Oxidative stress induced by chemotherapy may contribute to initiation and/or propagation of numerous alterations of blood platelet function. We determined the role of G-CSF in oxidative stress in blood platelets induced by cisplatin. This study provides evidence that G-CSF has a benefical effect on blood platelets. The exact mechanism of G-CSF action on blood platelets

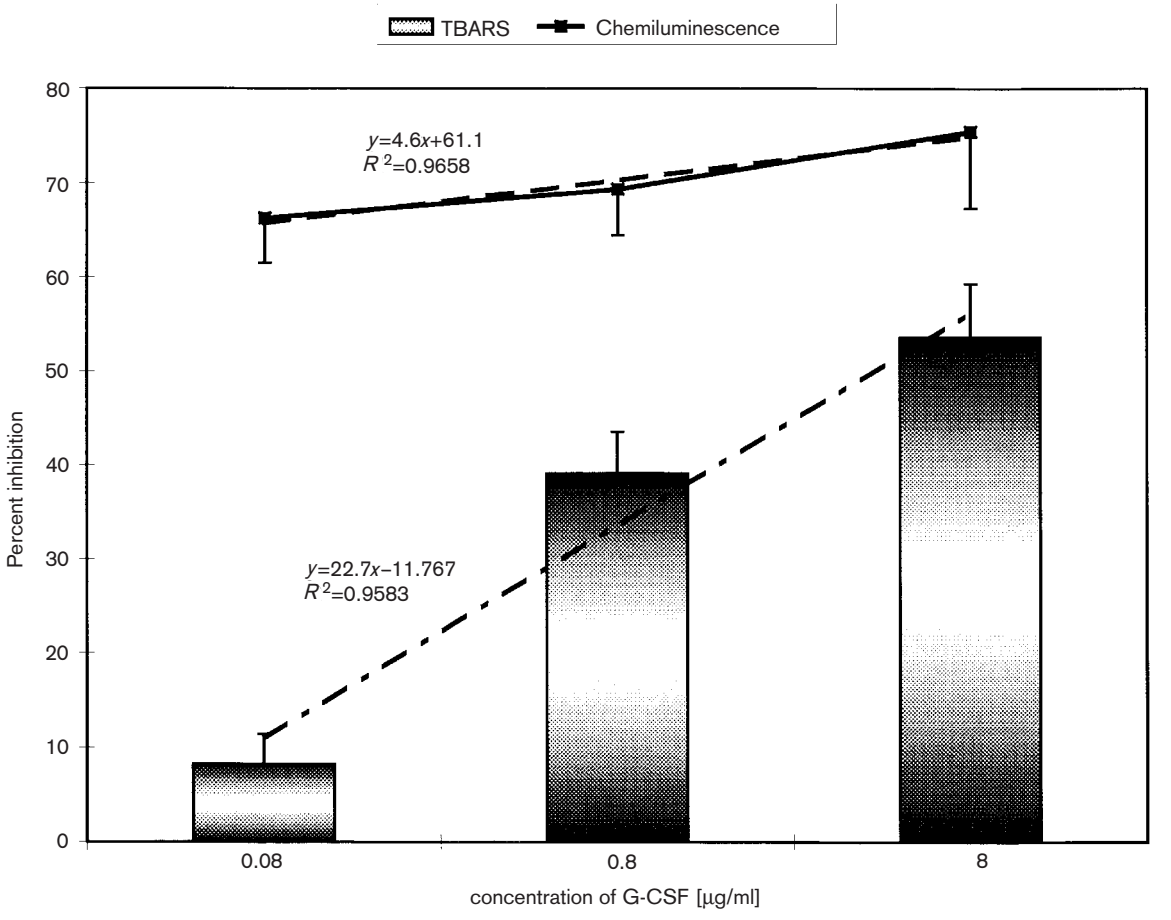

Figure 2. The dose-dependent inhibitory effects of G-CSF (0.08, 0.8 and 8 μg/ml; 5 min, 37°C) on the TBARS ($p<0.05$) and chemiluminescence ($p<0.05$). Results, expressed as percent of control, are means ± SD of four experiments.

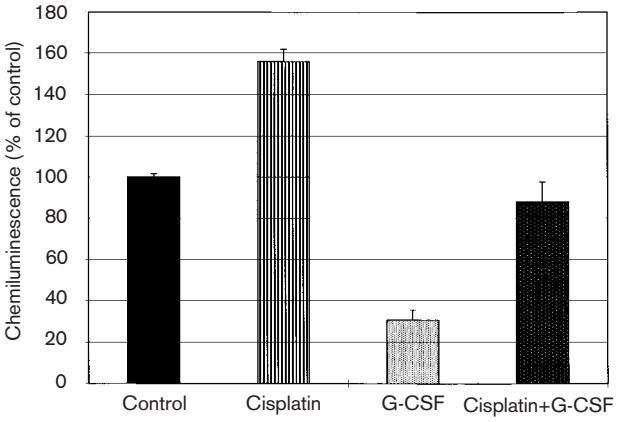

Figure 3. Production of ROS in control platelets and in platelets incubated with cisplatin alone (20 μM, 5 min, 37°C), G-CSF alone (0.8 μg/ml; 5 min, 37°C) and G-CSF together with cisplatin. Results, expressed as percent of control, are means ± SD of four experiments, *$p<0.05$, cisplatin-treated platelets versus control platelets, **$p<0.01$, G-CSF-treated platelets versus control platelets; ***$p<0.05$, cisplatin together G-CSF-treated platelets versus cisplatin-treated platelets.

remains unclear. Our preliminary results indicate that metabolism of arachidonate plays an important role in the interaction of G-CSF with platelets. This cytokine reduced thromboxane A_2 biosynthesis in thrombin-stimulated platelets when G-CSF was added to the whole blood. In combination with cisplatin, G-CSF decreased the inhibitory action of cisplatin on platelet endogenous arachidonate metabolism.[11] Our previous studies showed that, due to its reaction with free thiol groups, cisplatin induces the production of superoxide radicals in platelets.[5] It seems highly probable that the inhibitory action of G-CSF on free radical generation in blood platelets seen in our experiments (Figures 1 and 2) may correlate with the reduction of the endogenous arachidonate metabolism. In this study we demonstrate that G-CSF *in vitro* has an antioxidative effect on platelets and protects these cells against oxidative stress induced by spontaneous activation as well as by anticancer drug treatment (Figures 3 and 5). The level of free radicals seen in control platelets (in the absence of stimuli) was significant and seems to be caused by partial platelet activation during the isolation procedure. The pathway by which free radicals are

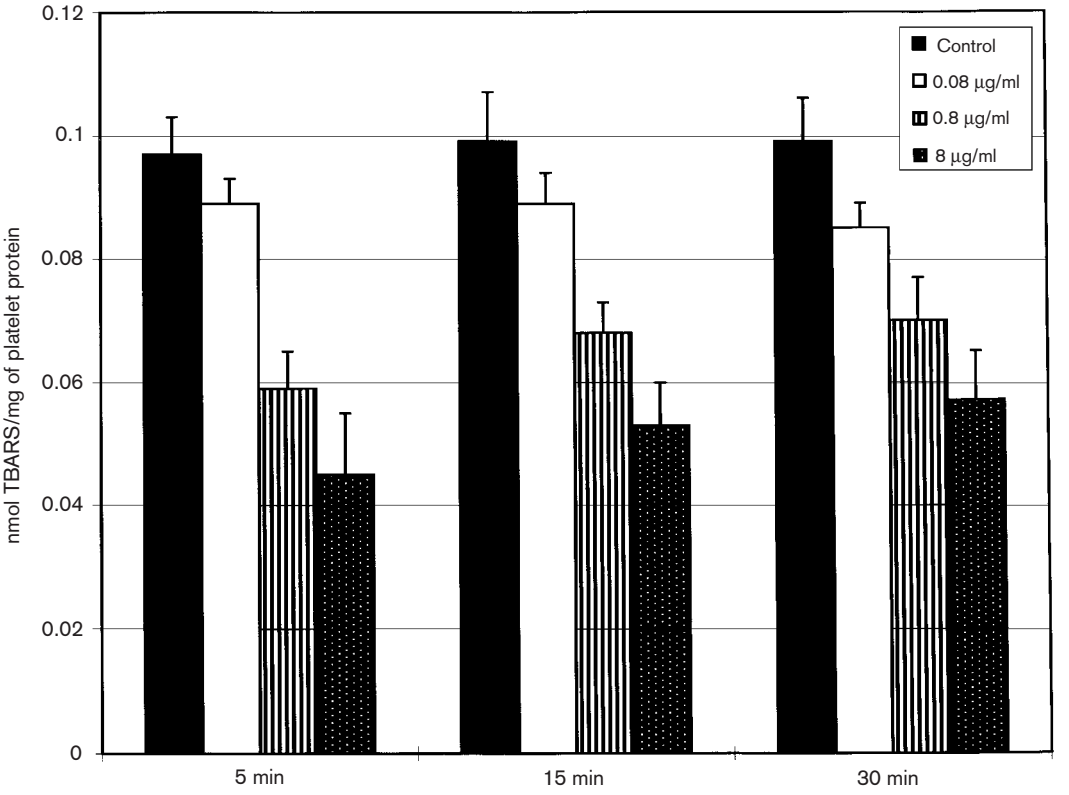

Figure 4. Dose- and time-dependent effects of G-CSF (0.08, 0.8 and 8 μg/ml; 5, 15 and 30 min, 37°C) on lipid peroxidation in pig isolated blood platelets. Results, expressed as percent of control, are means ± SD of six experiments, $p<0.05$ (compared with the control).

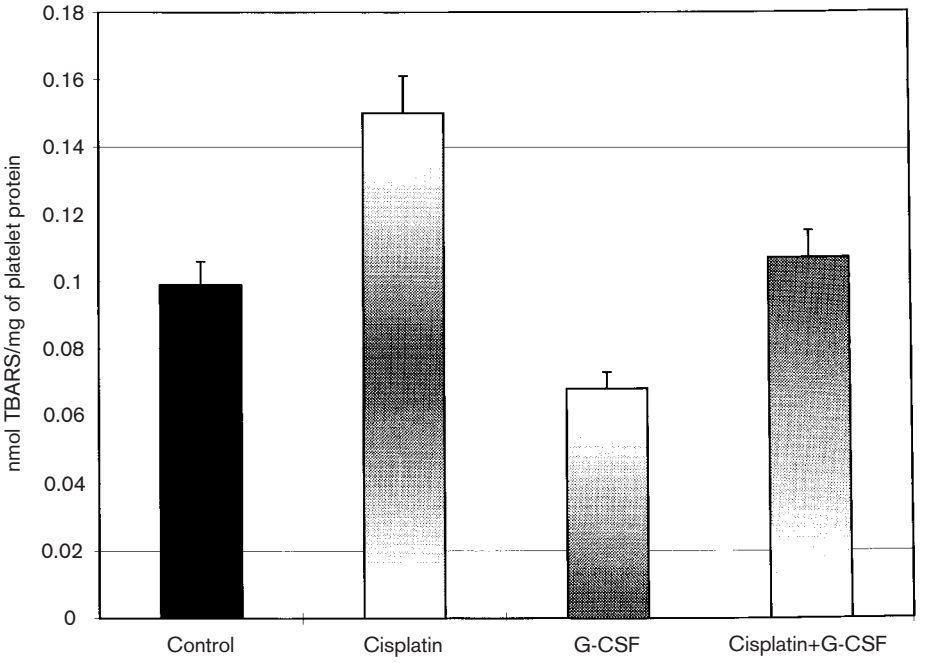

Figure 5. The effects of cisplatin alone (20 μM; 30 min, 37°C), G-CSF alone (0.8 μg/ml; 30 min, 37°C) and cisplatin together with G-CSF on lipid peroxidation in pig blood platelets. Results, expressed as percent of control, are means $\pm$ SD of six experiments, $^{*}p<0.05$, cisplatin-treated platelets versus control platelets, $^{**}p<0.01$, G-CSF-treated platelets versus control platelets, $^{***}p<0.05$, cisplatin together G-CSF-treated platelets versus cisplatin-treated platelets.

generated in blood platelets is not yet understood either. We noticed an increased level of superoxide radicals in activated platelets.[8] The results of Jahn and Hansch[27] have demonstrated that addition of exogenous arachidonic acid to platelets caused production of superoxide radicals during eicosanoid metabolism in the lipoxygenase pathway or via the phospholipase-dependent cleavage of platelet phospholipids. ROS may be also produced in the glutathione cycle. Formation of free radicals correlates with platelet activation. Oxidant species may behave as second messengers and participate in different steps of platelet activation (adhesion and aggregation).[28–32]

The toxic effects of cisplatin on the cells appear to be mediated in part by free radicals.[33] Our results indicate that cisplatin in platelets induces free radical generation, which is responsible for lipid peroxidation (Figures 3 and 5). G-CSF has a protective effect against cisplatin-induced changes in platelet metabolism (Figures 3 and 5) and may play a role as an antioxidant factor during spontaneous platelet activation. Moreover, it can prevent platelets being activated by cancer cells. Platelets from patients with cancer exhibit a variety of functional abnormalities. Many tumor cells can activate platelets via released ADP or generated thrombin.[34–36] Platelets participate not only in hemostasis, but are also involved in inflammation and metastasis. Activated platelets may initiate and propagate inflammation through the release of different biologically active substances such as PAF, serotonin, thromboxane A_2, cytokines and growth factors.[34–36] On the other hand, the changed platelet function in patients with cancer may result from anticancer drug treatment (cisplatin).[5,11] The presence of G-CSF during chemotherapy can protect blood platelets against the oxidative stress induced by drug treatment and reduce the toxic side effects of drugs on blood cells. G-CSF may have modulatory effects on oxidative stress induced by cancer cells in platelets. The potential clinical significance of G-CSF, its safety and its efficacy in chemotherapy are still unclear, and there is little information concerning its optimal dosage when used alone or in combination with chemotherapeutic agents. With the increased knowledge of multiple effects of G-CSF on different cells including blood platelets, it is expected that in the future carefully monitored clinical trials will provide more information regarding the benefits of G-CSF in cancer patients receiving cytotoxic chemotherapy. The efficacy of G-CSF therapy (related to the dosage) in protecting against changes of the hemostatic function of blood platelets in cancer patients caused by chemotherapeutic agents requires far greater investigation.

References

1. Lowenthal RM, Eaton K. Toxicity of chemotherapy. Paraneoplastic Syndromes. *Chem. Res Toxicol* 1996; **10**: 967–90.
2. Dabholkar M, Reed E. Cisplatin. *Cancer Chemother Biol Res Mod Ann* 1996; **7**: 88–100.
3. Wachowicz B, Olas B. Changes of platelet cytoskeleton induced by cisplatin in the relation to platelet activation. *Biomed Lett* 1995; **52**: 181–90.
4. Wachowicz B, Olas B. Comparative cytotoxicity of cisplatin, sodium selenite and selenium–cisplatin conjugate $[(NH_3)_2Pt(SeO_2)]$; changes of blood platelet activation. *Gen Physiol Biophys* 1997; **16**: 263–72.
5. Olas B, Wachowicz B. Cisplatin-induced changes of biological activity of blood platelets; thiol-related mechanisms. *Anti-Cancer Drugs* 1996; **7**: 476–82.
6. Olas B, Wachowicz B. Inhibitory effects of cisplatin and its conjugate with glutathione on blood platelet activity. *Platelets* 1997; **8**: 1–4.
7. Wachowicz B. Effect of cisplatin on lipid peroxidation in blood platelets. *Acta Biochim Pol* 1991; **38**: 87–90.
8. Wachowicz B, Szwarocka A. Response of pig blood platelets to cisplatin and sodium selenite: lipid peroxidation and oxygen radical generation. *Biomed Lett* 1994; **49**: 147–52.
9. Wachowicz B. Inhibitory effect of cisplatin on the enzymatic peroxidation of platelet endogenous arachidonate. *Cytobios* 1993; **75**: 157–62.
10. Wachowicz B, Kustroń J. Effect of cisplatin on lipid peroxidation in pig blood platelets. *Cytobios* 1992; **70**: 41–7.
11. Olas B, Wachowicz B, Wachowicz N. Effect of granulocyte-colony stimulating factor on pig blood platelets treated with cisplatin *in vitro*. *Anti-Cancer Drugs,* 1997; **8**: 204–9.
12. Tanaka R, Matsudaira T, Aizawa J, *et al*. Characterization of peripheral blood progenitor cells (PBPC) mobilized by filgrastin (rHuG-CSF) in normal volunteers: dose-effect relationship for filgrastim with the character of mobilized PBPC. *Br J Haematol* 1996; **92**: 795–803.
13. Brugger W, Birken R, Bertz H, *et al*. Peripheral blood progenitor cells mobilized by chemotherapy plus granulocyte-colony stimulating factor accelerate both neutrophil and platelet recovery after high-dose VP16, ifosfamide and cisplatin. *Br J Haematol* 1993; **84**: 402–7.
14. Kohno A, Takeyama K, Narabayashi M, *et al*. Low-dose granulocyte colony-stimulating factor enables the efficient collection of peripheral blood stem cells after disease-oriented, conventional-dose chemotherapy for breast cancer, malignant lymphoma and germ cell tumor. *Bone Marrow Transplant* 1995; **15**: 49–4.
15. Kreuser ED, Wadler S, Thiel E. Interactions between cytokines and cytotoxic drugs: putative molecular mechanisms in experimental hematology and oncology. *Semin Oncol* 1992; **19**: 1–7.
16. Tepler I, Elias L, Smith JW, *et al*. A randomized placebo-controlled trial of recombinant human interleukin-11 in cancer patients with severe thrombocytopenia due to chemotherapy. *Blood* 1996; **87**: 3607–54.
17. Shepherd FA, Goss PE, Rusthoven J, *et al*. Phase I trial of granulocyte-macrophage colony-stimulating factor with high-dose cisplatin and etoposide for treatment of small-cell lung cancer. *J Natl Cancer Inst* 1992; **84**: 59–70.
18. Sundman-Engberg B, Tidefelt U, Paul C. Effect of cytokine on the toxicity of cytostatic drugs on leukemic cells *in vitro* and *in vivo*. *Eur J Haematol* 1996; **56**: 1–6.
19. Vose JM, Armitage JO. Clinical applications of hematopoietic growth factors. *J Clin Oncol* 1995; **13**: 1023–35.
20. Gupta V, Jani JP, Emerson EO, *et al*. Modulation of cisplatin sensitivity and accumulation by interferon α-2A in human squamous carcinoma cell lines. *Int J Cancer* 1995; **63**: 419–22.
21. Basu A, Evans RW. Comparison of effects of growth factors and protein kinase C activators on cellular sensitivity to *cis*-diamminedichloroplatinum (II). *Int J Cancer* 1994; **58**: 587–91.
22. Wang ChH, Wang HM, Chen JS, *et al*. Intensive chemotherapy plus recombinant human granulocyte-colony stimulating factor support for distant metastatic nasopharyngeal carcinoma. *Oncology* 1997; **54**: 34–7.
23. Gordon MS, McCaskill-Stecens WJ, Battiato LA, *et al*. A phase I trial of recombinant human interleukin-11 (Neumega rhIL-11 growth factor) in women with breast cancer receiving chemotherapy. *Blood* 1996; **9**: 3615–24.
24. Smith JB, Ingerman CM, Silver MJ. Malondialdehyde formation as an indicator of prostaglandin production by human platelets. *J Lab Clin Med* 1976; **88**: 167–72.
25. Król W, Czuba Z, Scheller S, Gabryś J, Grabiec J, Shani J. Antioxidant property of ethanolic extract of propolis (EEP) as evaluated by inhibiting the chemiluminescence oxidation of luminol. *Biochem Int* 1990; **21**: 593–7.
26. Vatassary GT, Smith WE. Determination of α-tocopherolquinone (vitamin E quinone) in human serum. platelets, and red cell membrane samples. *Anal Biochem* 1987; **167**: 411–7.
27. Jahn B, Hansch GM. Oxygen radical generation in human platelets: dependence on 12-lipoxygenase activity and on the glutathione cycle. *Int Arch Allergy Appl Immun* 1990; **93**: 73–9.
28. Ambrosio G, Golino P, Pascucci I, *et al*. Modulation of platelet function by reactive oxygen metabolites. *Am J Physiol* 1994; **267**: 308–18.
29. Iuliano L, Pedersen JZ, Pratico D, Rotilio G, Violi F. Role of hydroxyl radicals in the activation of human platelets. *Eur J Biochem* 1994; **15**: 695–704.
30. Irani K, Pham Y, Coleman LD, *et al*. Priming of platelet $\alpha_{IIb}\beta_3$ by oxidants is associated with tyrosine phosphorylation of β_3. *Arterioscler Thromb Vasc Biol* 1998; **18**: 1698–706.
31. Forde RE, Fitzgerald DJ. Reactive oxygen species and platelet activation in reperfusion injury. *Circulation* 1997; **95**: 787–89.
32. Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, Violi F. Hydrogen peroxide is involved in collagen–induced platelet activation. *Blood* 1998; **91**: 484–90.
33. Boogaard PJ, Slikkerveer A, Nagelkerke JF, *et al*. The role of metallothionein in the reduction of cisplatin-induced nephrotoxicity by Bi^{3+}-pretreatment in the rat *in vivo* and *in vitro*. *Biochem Pharmacol* 1991; **41**: 369–75.
34. Goad KE, Gralnick HR. Coagulation disorders in cancer. *Complic Cancer* 1996; **10**: 457–84.
35. Poggi A, Rossi C, Beviglia L, *et al*. Platelet-tumor cell interactions. *Immunopharmacol Platelets* 1995; **8**: 151–65.
36. Wojtukiewicz M.Z, Tang DG, Ben-Josef E, *et al*. Solid tumor cells express functional tethered ligand thrombin receptor. *Cancer Res* 1995; **55**: 698–704.

(Received 3 November 1999; revised form accepted 18 November 1999)