Anti-Cancer Drugs 1997, 8, pp. 323–328

Enhanced immunostimulation by novel platinum anticancer agents

Heather J Muenchen, Surinder K Aggarwal, Hemant K Misra[1] and Peter J Andrulis[1]
Department of Zoology, Michigan State University, East Lansing, MI 48824-1115, USA. Tel: (+1) 517 353-2253; Fax: (+1) 517 432-2789. [1]Andrulis Pharmaceuticals Corp., Beltsville, MD 20705, USA.

'Poly-plat', SSP and SAP are second generation analogs of cisplatin which have been shown to activate murine peritoneal macrophages *in vivo* and *in vitro*. Murine peritoneal macrophages treated with 'poly-plat', SSP or SAP (5 μg/mg) for 2 h are stimulated to form cytoplasmic extensions. Drug-treated macrophages also elicit an increase in the number of lysosomes. In addition, analysis of tissue culture supernatants shows increased levels of interleukin-1α and tumor necrosis factor-α. These results show that 'poly-plat', SSP and SAP enhance the immune system with greater efficacy and potency than cisplatin.

Key words: Cisplatin, 'poly-plat', interleukin-1α, *in vitro*, lysosomes, macrophages, SAP, SSP, tumor necrosis factor-α.

Introduction

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP], a broad spectrum anticancer drug,[1] is proven to be effective in the treatment of testicular, ovarian, prostate, bladder, head and neck, and lung cancers.[2] Activated macrophages have been found to effectively destroy target cells by cytotoxic mechanisms.[3,4] The activation process includes the generation of extracellular products including interleukin (IL)-1α and tumor necrosis factor (TNF)-α.[5] Although cisplatin is an effective anticancer drug, it has very severe toxic side effects, of which gastrointestinal and nephrotoxicity are the dose-limiting factors.[6]

Poly-[(*trans*-1,2-diaminocyclohexane) platinum]-carboxyamylose ('poly-plat'), 5-sulfosalicylato-*trans*-(1,2-diaminocyclohexane) platinum (SSP) and 4-hydroxy-α-sulfonylphenylacetato (*trans*-1,2-diaminocyclohexane) platinum (II) (SAP) are second generation analogs of cisplatin with higher efficacy and potency, while eliciting less toxicity. This is particularly true of 'poly-plat' which contains one-fifth the platinum of cisplatin.[7] The possibility of drugs enhancing the immune system with less toxicity is very encouraging. We have here explored the effects of 'poly-plat', SSP and SAP on the macrophages so as to understand their mechanism(s) of action in enhancing the immune system.

Correspondence to SK Aggarwal

Materials and methods

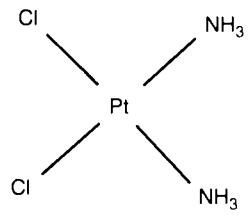
Cell cultures

Swiss webster mice (Charles River, Portage, MI) were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection of 5 ml chilled minimal essential medium (MEM; Gibco, Grand Island, NY) without serum containing 1% antibiotic–antimycotic [penicillin G (10 000 U/ml), streptomycin sulfate (10 000 μg/ml) and amphotericin B (25 μg/ml)] into the peritoneal cavity. After gently massaging the abdominal wall, cells were aspirated and seeded onto 18 mm^2 glass coverslips, placed in 35 mm Petri dishes at $2-4 \times 10^6$cells/ml and incubated for 2 h at 37°C after which cells were washed vigorously to remove non-adherent cells. Cell cultures were incubated in normal medium (minimal essential media and 10% heat-inactivated fetal calf serum) at 37°C in a 5% CO_2 incubator.

Treatments

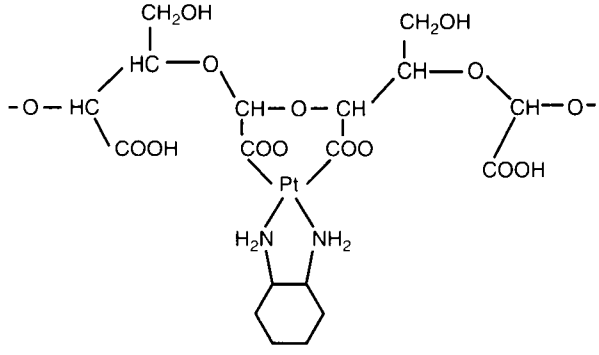
'Poly-plat' was prepared in 0.85% NaCl while SSP and SAP (Figure 1) were dissolved in 0.85% NaCl and 0.1% $NaCO_3$ in 5 μg/ml concentrations. Macrophages were treated with the drugs for 2 h. The drug(s)-containing medium was replaced by normal medium and supernatant (500 μl) was collected at 0.5, 1, 2 and 24 h for cytolytic factor(s) analysis. In addition, macrophages were also treated with cisplatin [5 μg/ml dissolved in physiological saline with 3 μl/ml of dimethylsulfoxide (DMSO; Sigma, St Louis, MO)]. Untreated cells in normal medium served as controls.

Cisplatin



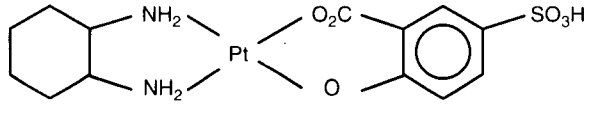
Cis-diamminedichloroplatinum(II)

'Poly-plat'



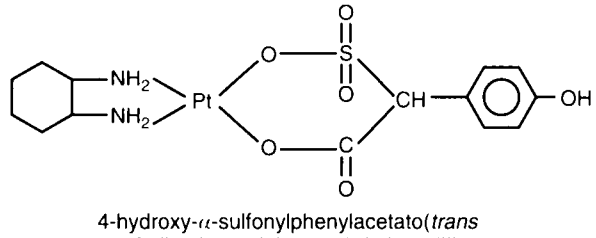
Poly-[(*trans*-1,2-diaminocyclohexane)platinum]-carboxyamylose

SSP



5-sulfosalicylato-*trans*-(1,2-diaminocyclohexane)platinum

SAP



4-hydroxy-α-sulfonylphenylacetato(*trans* 1,2-diaminocyclohexane)platinum(II)

Figure 1. Structure of compounds studied.

IL-1α assay

IL-1α was assayed using ELISA kits (Genzyme, Cambridge, MA). The method used the multiple antibody sandwich principle,[8] where monoclonal anti-murine IL-1α was used to bind murine IL-1α present in the supernatant. A biotinylated polyclonal antibody binding the IL-1α was added and unbound material was washed out. Peroxidase-conjugated avidin was used to bind these biotin tagged complexes. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with IL-1α (15–405 pg/ml) provided in the kits and linear regression analysis was performed.[9]

TNF-α assay

TNF-α released from supernatants of the macrophages was assayed using specific analysis kits (Genzyme). Again, the multiple antibody sandwich principle was utilized with a murine monoclonal antibody specific for murine TNF-α in the samples.[8] A horseradish peroxidase-conjugated anti-murine TNF-α antibody was used to bind the multiple epitopes on TNF-α. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with TNF-α (35–2240 pg/ml) provided in the kits and linear regression analysis was performed.[10]

Lysosomal assay

The quantitation of lysosomes before and after various treatments was achieved by exposing macrophage cultures to fresh medium containing acridine orange (5 μg/ml) for 30 min at 37°C in the dark.[11] After careful washing macrophages were examined under a Zeiss 10 laser scanning confocal microscope.

Results

IL-1α release

Compared to cisplatin treatments there was an increase in IL-1α levels in the supernatants of macrophages treated with either 'poly-plat', SSP or SAP (5 μg/ml) for up to 24 h of testing (Figure 2). The greatest increases were seen 2 h post-treament (400–500 pg/ml) with a subsequent decrease from there on. IL-1α levels demonstrated a consistent increase after cisplatin treament, reaching a maximum after 24 h. Although, IL-1α levels after 'poly-plat', SSP or SAP treament demonstrated a decline after a peak at 2 h, these levels were still equal to or above those after cisplatin treatment.

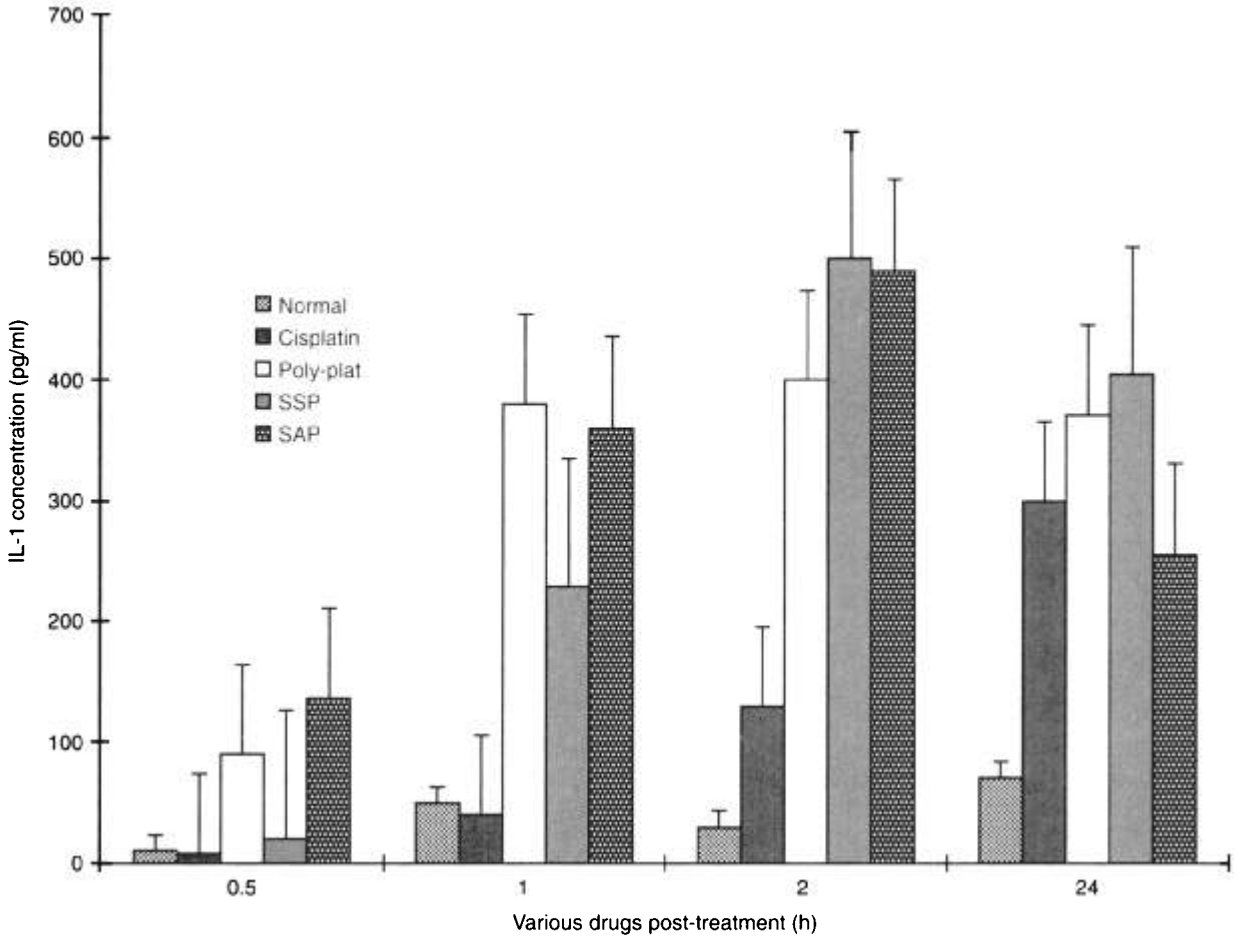


Figure 2. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages treated with either cisplatin, 'poly-plat', SSP, or SAP (5 μg/ml) after 30 min, and 1, 2 and 24 h post-treatment. Note the large increase at 2 h post-treatment in 'poly-plat', SSP and SAP. This is three times the amount of IL-1α released by cisplatin-treated macrophages.

TNF-α release

TNF-α increases were observed in SSP (5 μg/ml) at all times tested, with the most significant increase occurring at 2 h post-treatment (3250 pg/ml) (Figure 3). This level dramatically decreased at 24 h post-treatment (565 pg/ml). 'Poly-plat' and SAP (5 μg/ml) demonstrated very little TNF-α activity, barely reaching 200 pg/ml at 24 h post-treatment. Cisplatin demonstrated the usual enhanced release of TNF-α at various time intervals, reaching a peak value at 2 h post-treatment (300 pg/ml).

Macrophage activation

Murine peritoneal macrophages demonstrated extension formations after 2 h of treatment with 'poly-plat' and SSP (5 μg/ml) (Figure 4A and B). These cytoplasmic extensions radiate from the cell body in all directions. While SAP (5 μ1/ml)-treated macrophages did not show any extension formation, but instead assumed a discoid shape similar to that of the normal macrophages (Figure 4C and D). Cisplatin (5 μg/ml)-treated macrophages also showed similar extension formation but only after 24 h of treatment.

Lysosomal studies

Based on fluorescence measurements after acridine orange labeling, a 500-fold increase in the number of lysosomes in the macrophages after only 2 h of 'poly-plat' treatment (Figure 5A) was observed compared to normal cells (Figure 5B). The lysosomes were plentiful in the cytoplasm of the macrophages and in the drug-induced cytoplasmic extensions

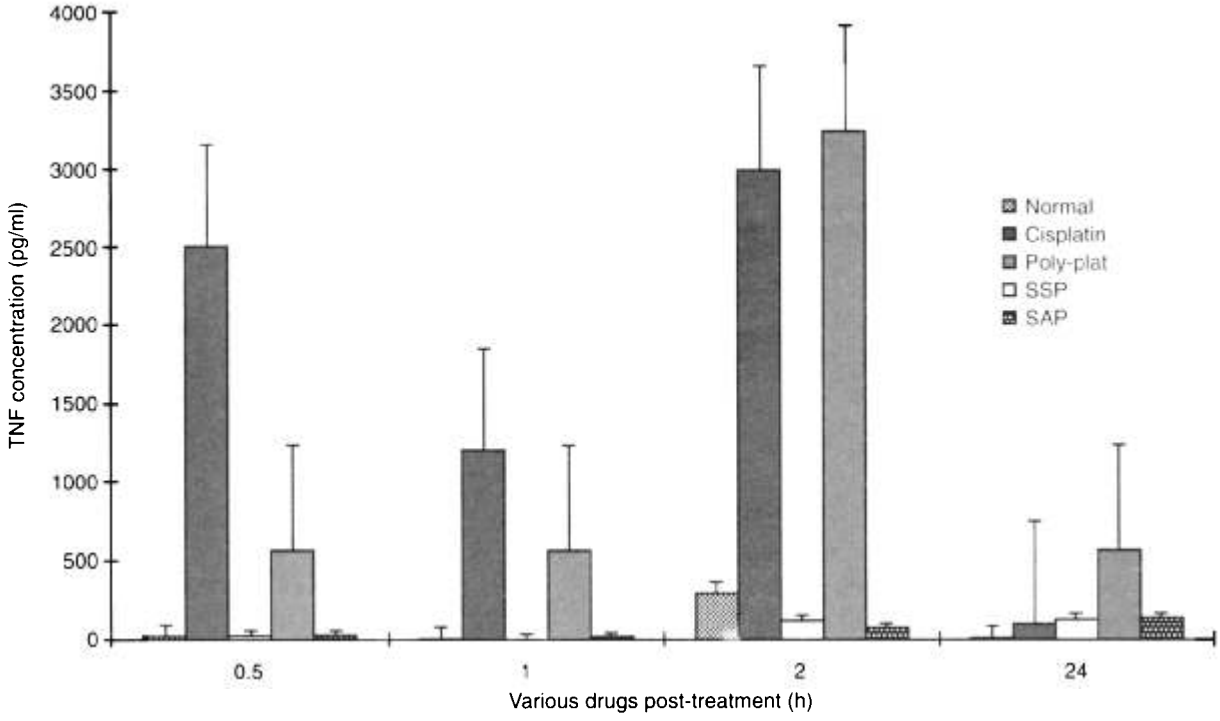


Figure 3. Bar graph showing TNF-α release in the supernatant of murine peritoneal macrophages treated with either cisplatin, 'poly-plat', SSP or SAP (5 μg/ml) after 30 min, and 1, 2 and 24 h post-treatment. Note the maximum increase in TNF-α for cisplatin and SSP 2 h post-treatment. Although, 'poly-plat' and SAP did show an increased level of TNF-α they were not significant compared to cisplatin or SSP treatments.

radiating from the cell body. SSP and SAP both demonstrated a 100-fold increase in the number of lysosomes. Comparatively, cisplatin treatment demonstrated only a 50-fold increase in the lysosomes (Figure 5C), both in the cell body and the cytoplasmic extensions.

Discussion

The anti-tumor agent cisplatin has been shown to enhance the immune system.[12] Cisplatin activates the macrophages by inducing an increase in their cytoplasmic lysosomes and promoting extension formations that establish contact with the tumor cells.[13] These macrophages lyse tumor cells through the transfer of lysosomes via the cytoplasmic extensions.[13] Cytolytic factors such as H_2O_2, superoxide anion, IL-1α, TNF-α, lysozyme and βNH have been demonstrated to be released from these treated macrophages.[5,14] Our study, using the second generation analogs of cisplatin, has shown that 'poly-plat', SSP and SAP elicit much more IL-1α, TNF-α, macrophage activation, as depicted by extension formations, and lysosomal increase compared to cisplatin.

Il-1α release by activated macrophages and its cytotoxicity to tumor target cells proves it to be potent mediator in tumor cell killing by macrophages.[14] Its release *in vitro* occurs in a cyclic manner showing its greatest increase at 2 h post-treatment after 'poly-plat', SSP and SAP. IL-1α can destroy tumor cells through several different pathways, i.e. production of superoxide, nitric oxide and hydrogen peroxide,[15,16] resulting in lipid peroxidation,[17] mitochondrial membrane depolarization and calcium mobilization, reduction in ATP synthesis,[18] and DNA base alterations.[19] Compared to cisplatin treatment, where the IL-1α release reaches a maximum at 24 h, these new drugs achieved it only after 60 min, reaching a maximum at 2 h. If the levels of IL-1α are any indication of the cytotoxicity then the efficiency of these drugs need to be tested in the *in vivo* system.

TNF-α also plays a regulatory role in inflammation and immunological response to tumors.[20] TNF-α activates production of nitric oxide which induces iron loss, and inhibits DNA synthesis, mitochondrial respiration and the citric acid cycle.[21,22] It is clear that TNF-α is an important mediator of tumor cell death by macrophages. Of the three analogs tested, only SSP demonstrated a rise in TNF-α production.

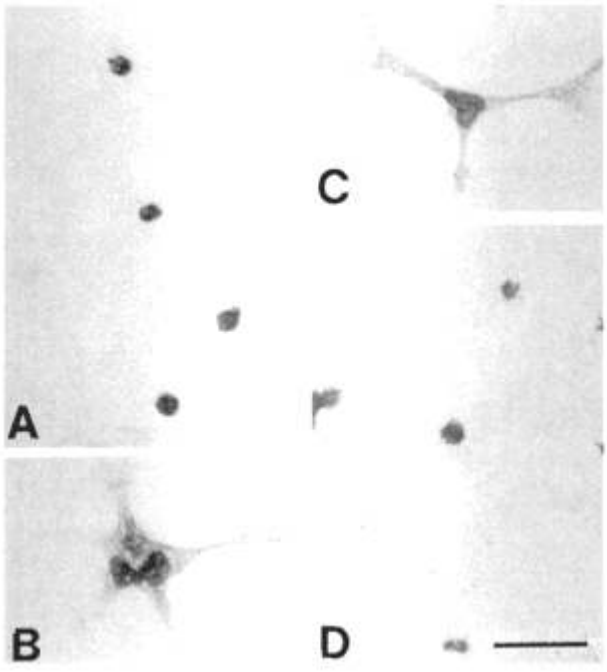


Figure 4. Light micrographs showing macrophages at 24 h in normal medium (A), and after 2 h of 'poly-plat' (B), SSP (C) and SAP (D). Note the extension formation after 'poly-plat' and SSP treatment(s). Only SAP assumes a discoid shape similar to that of untreated macrophages. Original magnification: ×1600. Bar = 0.5 mm.

Possibly the main thrust of SSP action is through increased release of TNF-α and IL-1α early enough compared to cisplatin.

Lysosomes take part in tumor cell death through macrophage activation.[23,24] Cisplatin-treated macrophages show an increase in their number of lysosomes and have been shown to be transferred to tumor cells through cytoplasmic extensions.[13] This results in the lysis of tumor cells.[25] A greater increase in lysosomes is seen in 'poly-plat'-, SSP- and SAP-treated macrophages when compared to cisplatin-treated macrophages. If lysosomal activity is any indication of cytotoxicity then 'poly-plat' may have greater efficacy in tumor cell death.

'Poly-plat' is curative in a range of solid tumors including renal, breast, ovarian, plasma cell myeloma and adenocarcinoma.[7] It has been shown to be up to 15 times as active as equimolar amounts of cisplatin, showing inhibition in many tumors where cisplatin is ineffective.[7] Recently, results have shown 'poly-plat' to have higher potency than cisplatin at the same level *in vitro* and *in vivo*.[7] This is due to 'poly-plat' having a molecular weight which is more than 10-fold higher than cisplatin.[7] 'Poly-plat', which contains only one-fifth the platinum of cisplatin, has also been shown to be less toxic.

SSP is curative of many cisplatin resistant tumors including M5 ovarian and several plasma cell myelomas. SSP also elicits no nephrotoxicity and is less toxic than cisplatin even at LD_{10} dose (180 mg/kg). *In vitro*, SSP treatment resulted in the greatest increase in IL-1α and TNF-α release.

From our *in vitro* studies it seems that SAP, unlike cisplatin, 'poly-plat' or SSP, does not function through the activation of the immune system. However, in *in vivo* studies, it has been shown to be most potent in the regression of X5563 plasma cell myeloma, M-5 ovarian carcinoma, 6C3HED lymphosarcoma, C3H mammary adenocarcinoma, B16 melanoma and DLD-2 human colon tumor xenografts. Compared to cisplatin's 9% inhibition of the DLD-2 human colon tumor xenografts, SAP has demonstrated a 99% inhibition. Whether its mechanism of action is through increased macrophage lysosomal activity or more effective DNA cross-linking remains to be explored.

Conclusion

'Poly-plat', SSP and SAP induce murine macrophage activation via the production of cytoplasmic extensions, lysosomes, and cytolytic factors IL-1α and

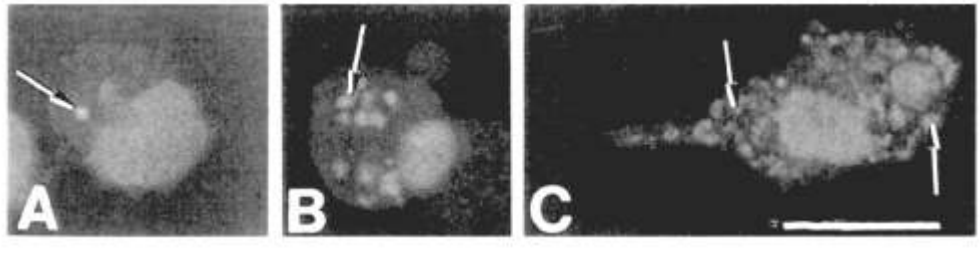


Figure 5. Fluorescent images taken from the Zeiss 10 laser scanning confocal microscope of macrophages labeled with acridine orange (5 μg/ml) showing lysosomal fluorescence in untreated (A), cisplatin (B) and 'poly-plat' (C). Note the large increase in lysosomal fluorescence after 'poly-plat' treatment. Original magnification: ×16 000. Bar = 10 μm.

TNF-α. Previous investigations have shown these drugs to be more effective than cisplastin *in vitro* and *in vivo* while eliciting less toxicity. Our studies support the activation of the immune system via the mediation of cytolytic factors and lysosomes as possible mechanisms of action of these drugs. Based on our observations we propose that 'poly-plat', SSP and SAP activate various cytolytic factors of the immune system better, compared to cisplatin.

References

1. Rosenburg B, VanCamp K, Krigas T. Inhibition of cell division on E. *coli* by electrolysis production from a platinum electrode. *Nature* 1965; **252**: 698.
2. Nicolini M. *Platinum and other metal coordination compounds in cancer chemotherapy.* Boston: Martinus Nijhoff; 1990.
3. Adams D, Hamilton T. Activation of macrophages for tumor cell kill: effector mechanisms and regulation. In Heppner GH, Fulton AM, eds. *Macrophages and cancer.* Boca Raton, FL: CRC Press 1988: 27.
4. Carleton S, Stevenson A, Hibbs J. Effector mechanisms for macrophage-induced cytostasis and cytolysis of tumor cells. In: Heppner GH, Fulton AM, eds. *Macrophages and cancer.* Boca Raton, FL: CRC Press 1988: 39.
5. Palma JP, Aggarwal SK. Cisplatin and carboplatin mediated activation of murine peritoneal macrophages *in vitro*: production of interleukin-1 and tumor necrosis factor. *Anti-Cancer Drugs* 1995; **6**: 1.
6. Walker E, Gale G. Methods of reduction of cisplatin nephrotoxicity, *Ann Clin Lab Sci* 1981; **11**: 397.
7. Fiebig HH, Dress M, Ruhnau T, Misra HK, Andrulis P, Hendriks HR. GB-21, a novel platinum complex with antitumor activity in human renal and mammary xenografts. *Proc Am Ass Cancer Res* 1996; **37**: 297.
8. Meager A. RIA, IRMA and ELISA assays for cytokines. In: Balkwill F, ed. *Cytokines: a practical approach.* Oxford: Oxford University Press 1991. 299.
9. Genzyme. *Elisa kit for quantification of mouse interleukin-1α.* Cambridge, MA: Genzyme Corp. 1994: 12.
10. Genzyme. *ELISA kit for quantification of mouse tumor necrosis factor-α.* Cambridge, MA: Genzyme Corp. 1993: 12.
11. Poole A. The detection of lysosomes by vital staining with acridine orange. In: Dingle J, ed. *Lysosomes: a laboratory handbook.*. Amsterdam: Elsevier/North Holland Biomedical 1977: 313.
12. Sodhi A, Singh S. Interaction between cisplatin treated macrophages and Dalton's lymphoma cells *in vitro*. *Exp Cell Biol* 1989; **56**: 1.
13. Palma JP, Aggarwal SK, Jiwa A. Murine macrophage activation after cisplatin or carboplatin treatment. *Anti-Cancer Drugs* 1992; **3**: 665.
14. Palma JP, Aggarwal SK. Cisplatin and carboplatin mediated release of cytolytic factors in murine peritoneal macrophages *in vitro*. *Anti-Cancer Drugs* 1994; **5**: 615.
15. Matsubara T, Ziff M. Increased superoxide anion release from human endothelial cells in response to cytokines. *J Immunol* 1986; **137**: 3295.
16. Zharazmi A, Neilsen, H, Bendtzen K. Recombinant interleukin-1 alfa and beta prime human monocytes superoxide production but have no effect on chemotaxis and oxidative burst response of neutrophils. *Immunobiology* 1988; **177**: 32.
17. Arouma O, Halliwell B, Dizdaroglu M. Iron ion dependent modification of bases in DNA by superoxide radical generating system hypoxanthine xanthine oxidase. *J. Biol Chem* 1989; **264**: 13024.
18. Richter C, Kass G. Oxidative stress in mitochondria: its relationship to cellular calcium homeostasis, cell death, proliferation and differentiation. *Chem-Biol Interact* 1991; **77**: 1.
19. Chong Y, Heppner G, Paul L, *et al.* Macrophage mediated induction of DNA strand breaks in target cells. *Cancer Res* 1989; **49**: 6652.
20. Balkwill F, Naylor M, Malik S. Tumor necrosis factor as an anticancer agent. *Eur J Cancer* 1990; **26**: 641.
21. Esumi H, Tannenbaum S. US–Japan Cooperative Cancer Research Program: seminar on nitric oxide synthase and carcinogenesis. Meeting Report. *Cancer Res* 1994; **54**: 297.
22. Knowles R, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994; **298**: 249
23. Bucana C, Hoyer L, Hobbs B, *et al.* Morphological evidence for translocation of lysosomal organelles from cytotoxic macrophages into the cytoplasm of tumor target cells. *Cancer Res* 1976; **36**: 4444.
24. Hibbs J. Heterocytolysis by macrophages activated by bacillus calmelte guerin: lysosome exocytosis into tumor cells. *Science* 1974; **148**: 468.
25. Singh S, Sodhi A. Interaction between cisplatin treated macrophages and Dalton's lymphoma cells. *in vitro*. *Exp Cell Biol* 1989; **56**: 1.

(Received 11 February 1997; accepted 18 February 1997)