Anti-Cancer Drugs 1997, **8**, pp. 853–858

Effects of cisplatin and taxol on inducible nitric oxide synthase, gastrin and somatostatin in gastrointestinal toxicity

Ying Wang and Surinder K Aggarwal
Department of Zoology, Michigan State University, East Lansing, MI 48824-1115, USA.
Tel: (+1) 517 353-2253; Fax: (+1) 517 432-2789.

Cisplatin (9 mg/kg) or taxol (20 mg/kg) treatment of Wistar rats produced a sharp decrease in inducible nitric oxide synthase (iNOS) and gastrin in the pyloric region of the stomach, and an increase in iNOS and somatostatin in the pancreatic islets. Nitric oxide (NO) functions as a relaxation factor in the smooth muscle of the muscularis mucosa while gastrin plays an important role in the gastroprotection of the mucosa through NO. It is proposed that a decline of the iNOS and gastrin after cisplatin or taxol treatments is related to distention of the stomach, and possibly nausea and vomiting. Hyperglycemia and glucose intolerance after cisplatin treatment may be caused by increases of somatostatin and iNOS in the pancreatic islets. Combination therapy with cisplatin and taxol seems to ameliorate various toxicities due to these two individual drugs.

Key words: Cisplatin, gastrin, iNOS, NO, pancreatic islet, somatostatin, stomach, taxol, ulceration.

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)], a broad-spectrum anticancer drug, has proven effective in the treatments of bladder, lung, ovarian,[1,2] head and neck,[3] testicular,[4] and breast[5,6] cancers and certain types of leukemia.[7] Drawbacks of this chemotherapeutic drug are its severe toxic side effects, which include nausea, vomiting, hyperglycemia,[8] stomach distention and gastric ulceration.[9,10] Nausea and vomiting are the dose-limiting factors in some cases. Distention of the stomach in rats[11] has been shown to parallel nausea and vomiting in other animals,[12] and seems to be a prerequisite for ulceration.[10] Nitric oxide (NO) has been shown to induce relaxation of the pyloric sphincter in the stomach.[13] NO and gastrin have been shown to generate gastroprotective effects against acid injury.[14,15] Somatostatin and NO suppress the secretion of insulin.[16] NO is regarded as one of the main mediators to induce insulin-dependent diabetes.[17]

Correspondence to SK Aggarwal

Taxol (Paclitaxel) is another antineoplastic drug with a taxane ring that shows significant anticancer activity. It is proven effective in the treatment of ovarian,[18,19] breast,[20] lung,[21] and head and neck[22] cancers. The adverse effects of this drug include nausea, vomiting, neutropenia and neurotoxicity. Nausea and vomiting are again the dose-limiting factors in some cases. Cisplatin and taxol in combination have proven to be much more effective and seemingly less toxic compared to either of the two drugs when administered alone. Based on the actions of NO and gastrin on the stomach and the functions of NO and somatostatin on the secretion of insulin from the pancreatic islets, the present study was undertaken to see the changes of gastrin and inducible nitric oxide synthase (iNOS) after cisplatin and taxol administration, and how these changes influence stomach motility and mucosa to cause nausea, vomiting and ulceration. Also, changes in somatostatin and iNOS after cisplatin treatment and what influence these have on pancreatic islets and insulin secretion were explored.

Materials and methods

Animals

Male Wistar rats (Charles Rivers, Wilmington, MA) weighing 100–150 g were kept on a 12 h light/12 h dark cycle with free access to laboratory animal feed and water. Cisplatin (9 mg/kg), taxol (20 mg/kg) and cisplatin plus taxol (9 mg/kg+20 mg/kg) were administered as i.p. injections in five divided dosages over a period of 5 days. The control animals received injection of the vehicle (0.85% NaCl) only. Twenty four hours following the last injection, the rats were sacrificed and various tissues excised.

Tissues

Stomach (cardia, body, pylorus) and pancreas were excised and fixed in Bouin's solution or 4% paraformaldehyde for 48 h. Fixed tissues were dehydrated and embedded in paraffin at 56°C. Sections (7 μm) were prepared for immunocytochemical studies.

Antisera

A monoclonal antibody specific for iNOS (Transduction Laboratories, Lexington, KY) was used in a dilution of 1:1000. The gastrin-specific (Dako, Carpinteria, CA) and somatostatin-specific (Incstar Stillwater, MN) antibodies were used in a dilution of 1:500.

Immunocytochemistry

Immunocytochemical staining was performed using the avidin-biotin-peroxidase complex method.[23] iNOS was demonstrated by the Vectastain Elite ABC Kit (Vector, Burlingame, CA), while somatostatin and gastrin were demonstrated using the Vectastain ABC-AP Kit.

iNOS, somatostatin and gastrin

Paraffin sections (7 μm) were treated with 3% H_2O_2 in dH_2O for 20 min to block any endogenous peroxidase activity and incubated in normal serum supplied in the kits for 20 min. These sections were incubated in the specific primary antibody overnight at 4°C and in the secondary antibody for 45 min at room temperature. Sections were subsequently incubated in the avidin-biotin-peroxidase complex for 45 min at room temperature. Peroxidase activity was revealed by incubation in 0.03% 3,3'diaminobenzidine (DAB) in 0.1 M sodium acetate/acetic buffer (pH 6.0), containing 2.5% nickel ammonium sulfate, 0.2% β-D-glucose, 0.04% ammonium chloride and 0.001% glucose oxidase.[24] Sections were rinsed in tap water for 5 min and 0.1 M acetate/acetic buffer (pH 6.0) before being mounted in permount through various dehydration series. For somatostatin and gastrin, alkaline phosphatase activity was revealed by using the Vector Red Substrate Kit (Vector Laboratories).

Results

High levels of iNOS activity were observed in the macrophagic cells at the base of the gastric villi and in the smooth muscle of the pyloric muscularis mucosa of the normal rat stomach (Figure 1). After cisplatin, taxol or cisplatin plus taxol treatments, iNOS positive cells were absent in the smooth muscle of the pyloric muscularis mucosa (Figure 2). However, at the base of the villi in the lamina propria, there were some iNOS positive cells, but their number was greatly reduced compared to the normals (Figure 2).

In the pancreas, iNOS positive cells were sparsely distributed throughout the islets (Figure 3). Cisplatin treatments induced an increase in their number and the intensity of their staining (Figure 4).

There were a large number of gastrin positive cells in the pyloric villi of the normal rat stomach (Figure 5), that were negative after cisplatin treatment (Figure 6). Taxol treatment induced a reduction in their number but maintained their presence (Figure 7). However, there were some gastrin positive cells at the base of

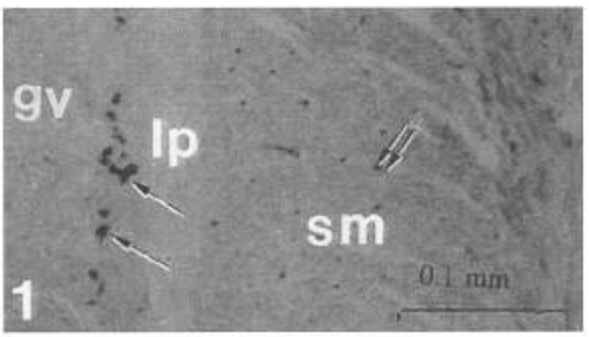

Figure 1. Cross-section of the muscularis mucosa from the pyloric region of a normal rat showing the presence of macrophagic cells (arrows) at the bottom of the gastric villi (gv) in the lamina propria and among the smooth muscle fibers (sm) of the muscularis mucosa (double arrows). Original magnification: ×262.

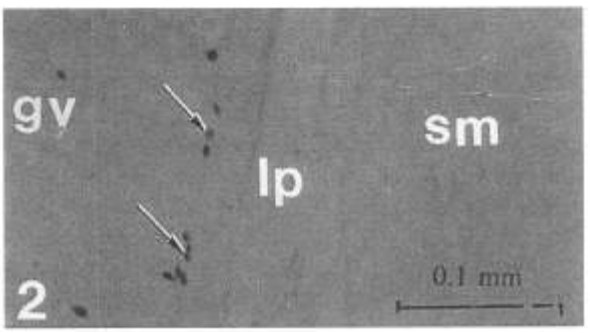

Figure 2. Pyloric region of a rat stomach after cisplatin treatment. Note the absence of macrophages from the muscularis mucosa (sm) and a much reduced number at the base of the villi (gv) in the lamina propria (lp). Original magnification: ×262.

the villi after cisplatin, taxol and cisplatin plus taxol not visible in the normal rat stomach (Figure 5). In their morphology and distribution these cells are similar to macrophagic cells.

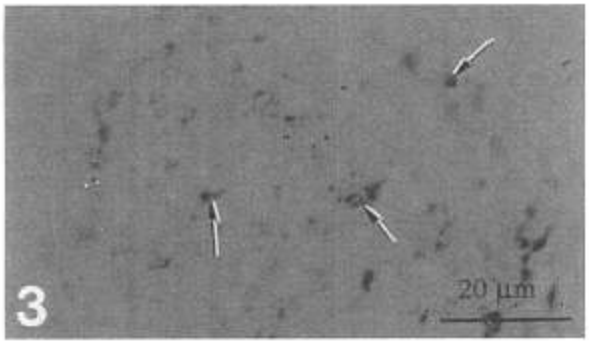

Figure 3. Random section through the pancreatic islet from a normal rat showing a sparse distribution of iNOS positive cells (arrows). Original magnification: × 1050.

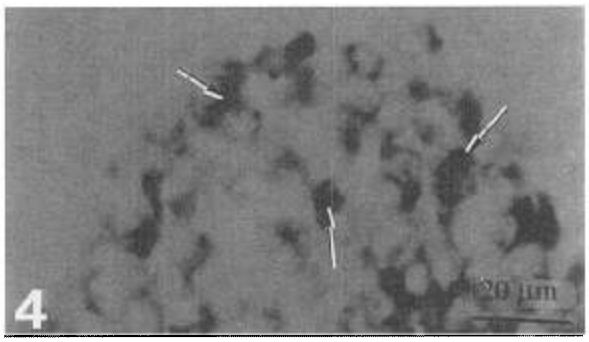

Figure 4. Pancreatic islet from a cisplatin-treated rat showing an increase in the number and staining intensity of cells due to iNOS. Original magnification: × 1050.

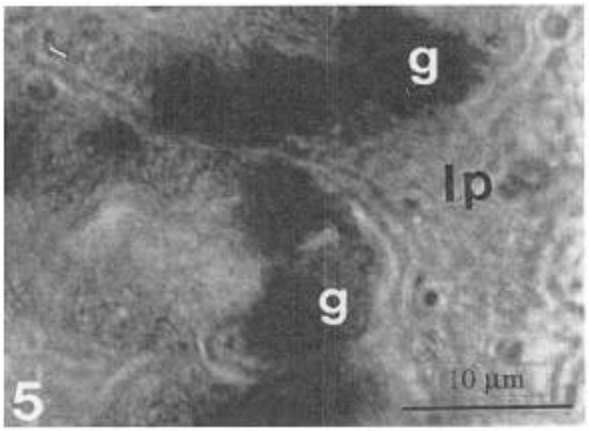

Figure 5. Gastrin positive cells (g) in the pyloric villi. Note the absence of gastrin positive cells from the lamina propria (lp). Original magnification: × 2625.

In the pancreas, somatostatin positive cells were located in the peripheral regions of the islets (Figure 8). After cisplatin treatment there was an increase in the number of such cells (Figure 9). Taxol treatment did not show any change from the normal. Cisplatin plus taxol treatment demonstrated an intermediate situation between the normal and cisplatin treatment.

Discussion

Various chemotherapeutic drugs, such as cisplatin and taxol, are known to induce nausea and vomiting, while cisplatin has also been shown to induce gastric lesions and ulceration in rats.[25] In order to combat these

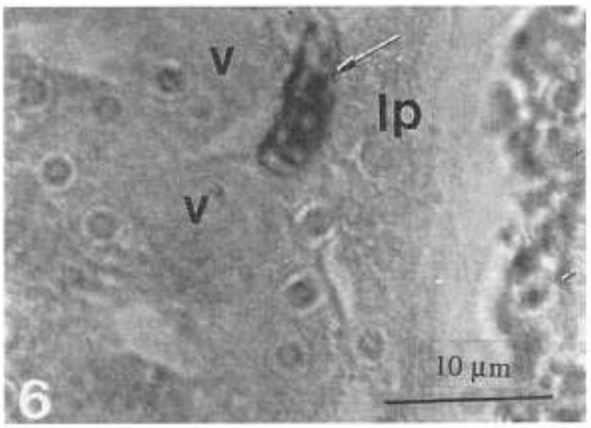

Figure 6. Pyloric villi (V) from a cisplatin-treated rat. Note the absence of gastrin positive cells from the villi. However, there are some gastrin positive (arrow) cells at the bottom of the villi in the lamina propria (lp). Original magnification: × 2625.

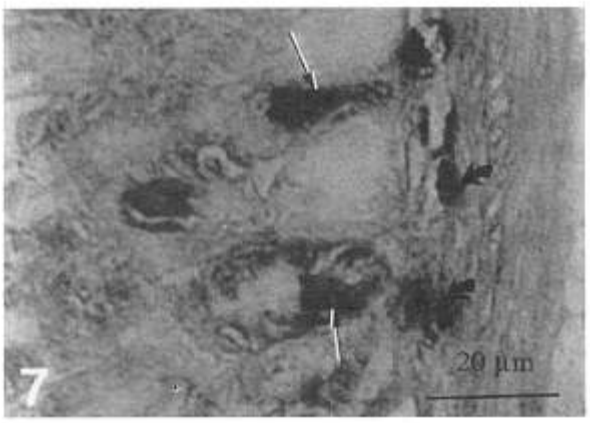

Figure 7. Distribution of gastrin positive cells both in the villi (arrows) and the lamina propria (curved arrows) after taxol treatment. Original magnification: × 1050.

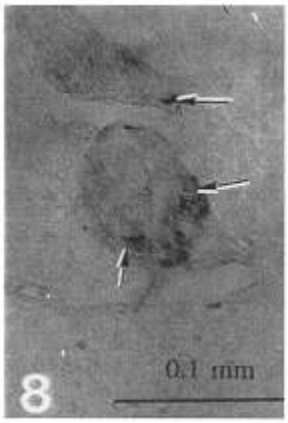

Figure 8. Somatostatin positive cells (arrows) in the pancraetic islets of a normal rat. Original magnification: ×262.

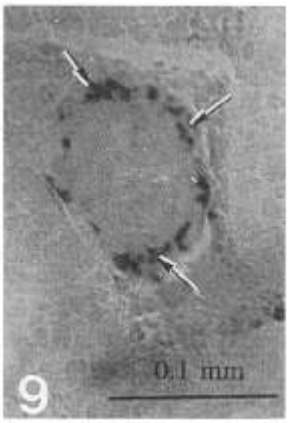

Figure 9. Increased number of somatostatin positive cells after cisplatin treatment (arrows). Original magnification: ×262.

adverse effects, it is essential to understand the cause or mechanism of induction of these effects. Distention of the stomach has been shown to parallel the nausea and vomiting that are associated with the clinical use of cisplatin.[11] In rats, bloating of the stomach has been demonstrated to be due to the retention of food in the stomach with lowered pH leading to ulceration.[10] Thus, alleviation of stomach distention may be the key point to the symptoms of nausea, vomiting and ulceration.

Normal stomach emptying depends on the contractility of the stomach and relaxation of the pyloric sphincter. The former is under the control of acetylcholine, while the latter is under NO control. A major part of the autonomic innervation of the gastrointestinal tract is by non-adrenergic non-cholinergic (NANC) neurons. In the sphincter region of the gut, this NANC innervation is mainly inhibitory and is physiologically important in the relaxation of the sphincters for the passage of the ingested food. NO and ATP contribute to the NANC inhibitory response of the rat pyloric sphincter.[13] Unlike the other neurotransmitters, NO can easily diffuse through the cell membrane.[26] NO produced in the pyloric smooth muscle can diffuse into the nearby sphincter muscle to cause its relaxation.

Release of acetylcholine from the nerve ending and NO production by the neuronal NO synthase (nNOS) are calcium dependent.[27] Cisplatin hydrolyzes into monoaqua and diaqua (divalent) species inside the cell under low chloride ion concentrations. In the diaqua configuration, it competitively binds to the calcium binding sites. Thus nNOS activation and acetylcholine vesicle release are prevented by a competitive inhibition of the calmodulin molecule. NO production and acetylcholine release are lowered after cisplatin treatment resulting in prolonged relaxation of the stomach smooth muscle,[10] and pyloric sphincter smooth muscle hypercontractility,[28] that in turn probably causes stomach distention.

In the normal rat pyloric sphincter smooth muscle and at the bottom of the pyloric villa, there are a large number of iNOS immunoreactive macrophages. However, after 2 days of cisplatin and taxol treatments, there are no iNOS immunoreactive macrophages in the pyloric smooth muscle. NO production from nNOS is calcium-calmodulin dependent, whereas NO production from iNOS is independent of calcium-calmodulin. Considering the different character of the iNOS from the nNOS, we can safely say that NO production by the iNOS is much less in cisplatin-treated rats compared to normal rats.

The main cause of gastric ulceration is the weakened gastroprotection, which includes maintenance of normal mucosal blood flow, mucosa integrity, mucosa growth and mucus secretion. Administration of L-NMMA (N^G-monomethyl-L-arginine), an inhibitor of NO synthase, induces gastric mucosal injury over a 45 min period in rats pretreated with indomethacin, in dosages of either agent that alone do not provoke mucosal injury. Likewise in rats chronically pretreated with capsaicin, L-NMMA induces extensive hemorrhagic mucosal injury. Such findings indicate that endogenous NO at least partly appears to serve as a modulator in the regulation of gastric mucosal integrity.[29,30] Endogenous NO may also play a role in offsetting the musosal microcirculatory actions of local vasoconstrictor mediators such as norepinephrine, neuropeptide or the endothelins. Through the increase of intracellular mediator (cGMP), NO induces mucus secretion without evidence of cellular da-

mage.[31] Mucus secreted by these epithelial cells plays a local protective role, since it forms a continuous viscoelastic layer that prevents access of pepsin to mucosa. Furthermore, secretion of bicarbonate into the unstirred layer of mucus can generate a gradient of increasing pH from the luminal bulk phase to the epithelial cell.[32] Lowered NO production after cisplatin treatment, which may be either from lowered iNOS or inhibited nNOS, probably accounts for the decrease of basal gastroprotection.

Gastrin, a kind of enteric peptide hormone, is released from the stomach and proximal portion of the gut upon normal ingestion of nutrients, and affects various physiological functions such as gastric secretion and mucosal growth.[33] It plays a physiological role in maintaining gastric mucosal integrity.[15] The protection induced by gastrin is accompanied by a significant elevation of gastric mucosal blood flow[15] which plays a crucial role in gastroprotection by prostaglandins[34] and endogenous NO release constitutively.[35,36] The pretreatment with N^{g}-nitro-L-arginine methyl ester significantly reduces the gastric blood flow induced by gastrin and prevents its protective activity. These effects of N^{g}-nitro-L-arginine methyl ester are reversed by concurrent administration of L-arginine, a substrate for NO synthase. NO plays a crucial role in mucosal hyperemia and gastroprotection by gastrin.[15] In cisplatin-treated rat stomachs, there was a sharp decline of gastrin-secreting G cells in the pyloric villi, compared to normal. This decline of gastrin may also be responsible for ulceration after cisplatin treatment. However, after taxol treatment there were some gastrin-secreting G cells along the pyloric villi. The number of such cells was small but sufficient to protect the mucosa, as no ulceration was observed after taxol treatment.

There are no gastrin immunoreactive cells in the pyloric region of the normal rat stomach other than the G cells along the villi. In the cisplatin and taxol-treated rat stomach, there are gastrin immunoreactive cells in the interstitial region along the bottom of the villi. These gastrin immunoreactive cells in the interstitial area of the pyloric region may be compensatory for the sharp decline of gastrin-secreting G cells after cisplatin and taxol treatments.

Similar to other heavy divalent metal ions, i.e. cadmium,[37] cobalt,[38] zinc[39] and nickel,[40] cisplatin administration impairs glucose tolerance, which is indicated by marked hyperglycemia.[8] Administration of cisplatin results in hyperglucagonemia and a relative deficiency in insulin secretion. Timed interval studies of glucose intolerance following cisplatin treatment have been demonstrated to be related to a deficiency in insulin secretion.[8,41,42]

Somatostatin-14 and somatostatin-28 are the two known bioactive peptides, and share the ability to inhibit the release of various peptides, including insulin,[43] which is mediated by a pertussis toxic-sensitive GTP-binding protein.[16] Thus, in the cisplatin-treated rat pancreatic islets, the increased somatostatin-immunoreactive cells may be responsible for the suppression of insulin.

There is a marked increase in iNOS immunoreactive cells in the rat pancreatic islets after cisplatin treatment. Both β cells and macrophages in pancreatic islets may be sources of iNOS.[44] In insulin-dependent diabetes, a large amount of NO produced by the increased number of iNOS in pancreatic islets suppresses insulin secretion and participates in the destruction of β cells.[45]

In conclusion, cisplatin or taxol depress iNOS and gastrin levels in the stomach while increasing iNOS and somatostatin in the pancreatic islets. In combination they seem to modulate these effects probably through their different mechanisms of action.

References

1. Comis RL. Cisplatin: the future. *Semin Oncol* 1994; **21:** 109-13.
2. Ozols RF, Young RC. Chemotherapy of ovarian cancer. *Semin Oncol* 1984; **11:** 251-7.
3. Choksi A, Hong WK. Chemotherapy of head and neck cancer. In: Nicolini M, ed. *Platinum and other metal coordination compounds in cancer chemotherapy.* Padua: Martinus Nijhoff 1987: 375-85.
4. Einhorn LH, Williams SD. Chemotherapy of disseminated testicular cancer. *Cancer* 1980; **46:** 1339-44.
5. Fan S, Smith ML, Rivet DJ, *et al.* Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Res* 1995; **55:** 1649-54.
6. Jurga L, Misurova E, Kovac V, Sevcikova L. The role of cisplatin in chemotherapy of advanced breast cancer. *Neoplasma* 1994; **41:** 347-52.
7. Miller AA, Hargis JB, Lilenbaum RC, Fields SZ, Rosner GL, Schilsky RL. Phase I study of topotecan and cisplatin in patients with advanced solid tumors: a cancer and leukemia group B study. *J Clin Oncol* 1994; **12:** 2743-50.
8. Goldstein RS, Mayor GH, Gingerich RL, Hook JB, Rosenbaum RW, Bond JT. The effects of cisplatin and other divalent compounds on glucose metabolism and pancreas endocrine function. *Toxicol Appl Pharmacol* 1983; **69:** 432-41.
9. Aggarwal SK. A histochemical approach to the mechanism of action of cisplatin and its analogues. *J Histochem Cytochem* 1993; **41:** 1053-73.
10. Aggarwal SK, Antonio JDS, Sokhansanj A, Miller CM. Cisplatin induced peptic ulcers, vagotomoy, adrenal and calcium modulation. *Anti-Cancer Drugs* 1994; **5:** 177-93.
11. Roos IA, Fairlie DP, Whitehouse MW. A peculiar toxicity manifested by platinum(II) amines in rats: gastric distension after intraperitoneal administration. *Chem-Biol Interact* 1981; **35:** 111-7.

12. Cubeddu LX, Lindley CM, Wetsel W, Carl PL, Negro-Vilar A. Role of angiotensin II and vasopressin in cisplatin-induced emesis. *Life Sci* 1990; **46:** 699–705.
13. Soediono P, Burnstock G. Contribution of ATP and nitric oxide to NANC inhibitory transmission in rat pyloric sphincter. *Br J Pharmacol* 1994; **113:** 681–6.
14. Stroff T, Lambrecht N, Peskar BM. Nitric oxide as mediator of the gastroprotection by cholecystokinin-8 and pentagastrin. *Eur J Pharmacol* 1994; **260:** R1–2.
15. Konturek SJ, Brzozowski T, Bielanski W, Schally AV. Role of endogenous gastrin in gastroprotection. *Eur J Pharmacol* 1995; **278:** 203–12.
16. Nilsson T, Arkhammar P, Rorsman P, Berggren PO. Suppression of insulin release by galanin and somatostatin is mediated by a G-protein. *J. Biol Chem* 1989; **264:** 973–80.
17. Corbett J, Mikhael A, Shimizu J, *et al.* Nitric oxide production in islets from nonobese diabetic mice: aminoguanidine-sensitive and -resistant stages in the immunological diabetic process. *Proc Natl Acad Sci USA* 1993; **90:** 8992–5.
18. Sarosy G, Kohn E, Stone D, *et al.* Phase 1 study of taxol and granulocyte colony-stimulating factor in patients with refractory ovarian cancer. *J Clin Oncol* 1992; **10:** 1165–70.
19. Thigpen J, Blessing J, Ball H, Hummel S, Barrett R. Phase 11 trial of paclitaxel in patients with progressive ovarian carcinoma after platinum-based chemotherapy. *J Clin Oncol* 1994; **12:** 1748–53.
20. Holmes F, Walters R, Theriault R, *et al.* Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. *J Natl Cancer Inst* 1991; **83:** 1797–805.
21. Murphy W, Fossella F, Winn R, *et al.* Phase II study of taxol in patients with untreated advanced non-small cell lung cancer. *J Natl Cancer Inst* 1993; **85:** 384–8.
22. Forastiere A, Neuberg D, Taylor S, Deconti R, Adams G. Phase II evaluation of taxol in advanced head and neck cancer: an Eastern Cooperative Oncology Group trial. *Monogr Natl Cancer Inst* 1993; **15:** 181–4.
23. Hsu S, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABS) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981; **29:** 577–80.
24. Shu S, Ju G, Fan L. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 1988; **85:** 169–71.
25. Aggarwal SK, Fadool JM. Cisplatin and carboplatin induced changes in the neurohypophysis and parathyroid, and their role in nephrotoxicity. *Anti-Cancer Drugs* 1993; **4:** 149–62.
26. Kendel ER, Schwartz JH, Jessell TM. *Principles in neural science.* Norwalk, CT: Appleton and Lange 1991.
27. Jarve R, Aggarwal S. Cisplatin-induced inhibition of the calcium-calmodulin complex, neuronal nitric oxide synthase activation and their role in stomach distention. *Cancer Chemother Pharmacol* 1997; **39:** 341–8.
28. Huang PL, Dawson TM, Bredt DS, Synder SH, Fishman MC. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 1993; **75:** 1273–86.
29. Whittle BJR. Neuronal and endothelium-derived mediators in the modulation of the gastric microcirculation: Integrity in the balance. *Br J Pharmacol* 1993; **110:** 3–17.
30. Whittle BJR, Lopez-Belmonte J, Moncada S. Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. *Br J Pharmacol* 1990; **99:** 607–11.
31. Brown J, Keates A, Hanson P, Whittle B. Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. *Am J Physiol* 1993; **265:** G418–22.
32. Allen A, Hunter AC, Leonard AJ, Pearson JP, Sellers LA. Peptic activity and the mucus-bicarbonate barrier. In: Garner A, Whittle BJR, eds. *Advances in drug therapy of gastrointestinal ulceration.* Chichester: John Wiley 1989: 139–55.
33. Walsh JH. Gastrin. In: Walsh JH, Graham GJ, eds. *Gut peptides: biochemistry and physiology.* New York: Raven Press 1994: 75–122.
34. Leung FN, Robert A, Guth PH. Gastric mucosal blood flow in rats after administration of 16,16-dimethyl prostaglandin E2 at a cytoprotective dose. *Gastroenterology* 1985; **88:** 1948–53.
35. Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988; **333:** 664–6.
36. Brown JF, Tepperman BL, Hanson PJ, Whittle BJR, Moncada S. Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. *Biochem Biophys Res Commun* 1992; **184:** 680–5.
37. Ghafghazi T, Mennear JH. Effects of acute and subacute cadmium administration on carbohydrate metabolism in mice. *Toxicol Appl Pharmacol* 1973; **26:** 231–40.
38. Eaton RP. Glucagon secretion and activity in the cobalt chloride-treated rat. *Am J Physiol* 1973; **225:** 67–72.
39. Horak E, Sunderman FWJ. Effects of Ni(II) upon plasma glucagon and glucose in rats. *Toxicol Appl Pharmacol* 1975; **33:** 388–91.
40. Clary JJ. Nickel-chloride induced metabolic changes in the rat and guinea pig. *Toxicol Appl Pharmacol* 1975; **31:** 55–65.
41. Goldstein RS, Mayor GH, Rosenbaum RW, Hook JB, Santiago JV, Bond JT. Glucose intolerance following cisplatinum treatment in rats. *Toxicology* 1982; **24:** 273–80.
42. Fradkin J, Shamoon H, Felig P, Sherwin RS. Evidence for an important role of changes in rather than absolute concentrations of glucagon in the regulation of glucose production in humans. *J Clin Endocrinol Metab* 1980; **50:** 698–703.
43. Patel YC. Somatostatin. In: Becker KL, ed. *Principles and practice of endocrinology and metabolism.* Philadelphia, PA: Lippincott 1994: 1436–45.
44. Rabinovitch A, Suarez-Pinzon WL, Sorsensen O, Bleackley RC. Inducible nitric oxide synthase (iNOS) in pancreatic islets of nonobese diabetic mice: identification of iNOS-expressing cells and relationships to cytokines expressed in the islets. *Endocrinology* 1996; **137:** 2039–99.
45. Mandrup-Poulsen T, Helqvist S, Wogensen LD, *et al.* Cytokines and free radicals as effector molecules in the destruction of pancreatic beta cells. *Curr Top Microbiol Immunol* 1990; **164:** 169–93.

(Received 31 July 1997; accepted 7 August 1997)