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The antitumour effect and toxicity of *cis*-platinum and *N*-methylformamide in combination

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Summary. *N*-Methylformamide (NMF), currently undergoing phase II clinical evaluation for the treatment of cancer, and the established antitumour agent *cis*-platinum (CDDP) have nonoverlapping toxicities, with the exception of gastrointestinal side effects. The major target organs for the toxicities of the compounds are the liver (NMF) and the kidney (CDDP). Furthermore, NMF is nonleukopenic. In view of this, and of recent evidence that NMF enhances the cytotoxic effect of CDDP in vitro the activity of NMF and CDDP against the M5076 sarcoma implanted in mice was investigated, together with the various toxicities in mice and rats. The antitumour effect of NMF in combination with CDDP was additive, but NMF did not alter the leukopenia produced by CDDP in the tumour-bearing mice. CDDP produced only a minimal increase in the hepatotoxicity of NMF in mice, and NMF did not augment the nephrotoxicity of CDDP in rats (except for a small effect on calcium excretion). The results support suggestions that clinical evaluation of combination chemotherapy with NMF and CDDP is warranted.

Introduction

Combination chemotherapy is often used in the treatment of patients with cancer. The aim of co-administering drugs is to achieve a therapeutic advantage over single-agent therapy, for example by combining drugs with nonoverlapping toxicities.

N-Methylformamide (NMF) is currently undergoing phase II clinical evaluation for the treatment of cancer. NMF has marked antineoplastic activity against both murine tumours and human xenografts implanted in mice [3, 7] and showed modest antitumour activity in a recent phase I clinical trial [18]. Although the most frequent side effect of NMF in man is anorexia with nausea [18], hepatotoxicity has been regarded as dose-limiting [18, 19]. NMF did not cause leukopenia in either animals [21] or man [18]. This has aroused interest in its potential for combination with other antitumour agents, for many of which (e.g., cyclophosphamide) leukopenia can prove dose-limiting. Studies in mice indicated that NMF and cyclophosphamide given in combination had an additive antitumour effect, but neither hepatotoxicity nor leukopenia was greater than when NMF or cyclophosphamide was administered alone [12].

Dexter et al. [8] found that NMF enhanced the in vitro activity of *cis*-platinum (CDDP) against two human cancer cell lines, DLD-1 (clone D) and HCT-15, and suggested that the antitumour efficacy of the combination of NMF and CDDP should be assessed further. The toxicities of CDDP include gastrointestinal, neurological, and haematological effects, and each of these may be dose-limiting in some patients. Although CDDP-induced nephrotoxicity can be minimized by rigorous hydration and the concomitant use of diuretics, it is widely considered to be the major toxicity with this drug.

In the present study we have examined the efficacy of the combination of NMF and CDDP against the murine M5076 sarcoma and have studied the various toxicities of the combination relative to the single agents.

Materials and methods

Animals. Female BDF_1 (DBA/2 × C57/BL) mice (18–23 g) and male Fischer 344 rats (100–160 g) were purchased from Bantin and Kingman Ltd, Hull, UK. Mice and rats were fed on Pilsbury's breeding diet (modified 41B) and allowed tapwater ad libitum.

Drugs. NMF was obtained from Aldrich Chemicals, UK, CDDP from Johnson Matthey. NMF was purified further by redistillation. Drugs were administered dissolved in saline to mice or rats.

Antitumour assays. The M5076 sarcoma was maintained as a solid subcutaneous tumour in BDF_1 mice and passaged every 21 days. For the antitumour assays, fragments of the M5076 sarcoma were first obtained from donor mice. These were pooled, homogenized and diluted with saline to produce a suspension of 10^7 cells/ml. Cells (10^6 in 0.1 ml) were then injected IM into the left hind legs of groups of 5 or 10 female BDF_1 mice. Drugs were administered IP according to the schedules described in Tables 1 and 2. In the drug combination study, treatment was delayed until day 12 after implant, since the tumours are both palpable and measurable on this day. Mean tumour volumes were determined in the following manner. Tumour diameters were first measured by calipers and the volumes were calculated from the formula

$$\text{Volume} = \frac{l \times w^2}{2},$$

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where l represents the longest tumour diameter and w the diameter perpendicular to this axis. ID_{90} refers to that dose which will produce 90% inhibition of the mean tumour volume in treated mice compared with the mean volume in control mice. Lethal dose values were determined by using a range of doses varying from nonlethal to 100% mortality in mice bearing tumours. LD_{10} and LD_{50} values were calculated according to the method of Litchfield and Wilcoxon [15]. The therapeutic index was calculated as LD_{10}/ID_{90}.

Leukopenia. While mice bearing M5076 tumours were under halothane/nitrous oxide anaesthesia, blood samples were collected from the tip of the tail into blood cell pipettes. Blood was diluted in a 1% acetic acid/saline solution and stained with gentian violet, after which leukocytes were counted with a Weber B.S.A. improved Neubauer haemocytometer.

Hepatotoxicity. In this study female BDF_1 mice without tumours were used, since the destruction of tumours by the drugs might have contributed to elevated enzyme levels in the plasma of these mice. While mice were under halothane/nitrous oxide anaesthesia blood samples (1 ml) were collected into heparinized syringes by exsanguination from the abdominal aorta at the iliac bifurcation. Immediately after this procedure the animals were killed. Plasma was obtained by centrifugation for 1 min in a Beckman microfuge. Sorbitol dehydrogenase (SDH) activities in the plasma were assayed by a method described by Rose and Henderson [23], while L-alanine aminotransferase (ALT) and L-aspartate aminotransferase (AST) were assayed according to methods described by Kachmar and Moss [11]. All enzyme activities were assayed at 37 °C.

Nephrotoxicity. Dosage schedules. In most studies of the nephrotoxicity of CDDP in animals the drug has been administered as a single large dose. With regard both to an optimal antitumour dosing schedule in mice (Table 1) and to clinical dosing schedules, we thought it more pertinent to administer several small doses at intervals of a few days. The chosen dose of CDDP, 2 mg kg^{-1}, was similar to that used in man on a per unit of body weight basis. This dose was considered unlikely to cause mortality in rats but likely to produce submaximal renal damage, so that any augmentation by NMF would be clearly seen. The dosing schedule of NMF was the same as that used in mice in the antitumour and hepatotoxicity studies. However, in view of the unknown sensitivity of the Fischer 344 rat to the hepatotoxic effect of NMF, the unit doses were reduced to 100 mg kg^{-1}, a dose which caused minimal hepatotoxicity when injected in five daily doses to Balb/c mice [27], although not to BDF_1 mice.

The rats were randomized into three treatment groups ($n=6$) which received CDDP alone, NMF alone, or CDDP and NMF. The drugs were administered by IP injection at 10.00 a.m. The first day of drug administration was designated day 0. CDDP, 2 mg kg^{-1}, in a dose volume of 10 ml kg^{-1} was injected on days 0, 4, 8, and 12. NMF, 100 mg kg^{-1}, in a dose volume of 1.0 ml kg^{-1} was given in ten daily injections from days 3 to 12. Each injection of CDDP or NMF was controlled by administration of an equivalent volume of saline to a fourth group of six rats. The rats which received only one drug received an injection of saline on each occasion when the second drug was given to the combination group. All rats had free access to tapwater throughout the study, and the CDDP+NMF group had free access to food. During the dosing period and for 4 subsequent weeks the food consumption of the other three groups was regulated, on a daily basis, to match that of the CDDP+NMF group. From day 42 all groups had unrestricted access to food. Body weight and water intake were monitored daily.

Collection and analysis of urine. The study was conducted in an environmentally controlled room (12 h light, 08.00–20.00 at 22 °C). Rats were acclimatized to individual metabolic cages for 4 days before urine collection was started. Spontaneously voided urine, essentially free of contamination by faeces or food, was collected over 24-h periods commencing at 10.00 a.m. Collection tubes were covered and surrounded by ice to prevent evaporation and denaturation of enzymes.

Two control 24-h urine collections were made from each group on days −4 and −1. Urine was then collected every other day during the dosing period and subsequently at intervals of increasing length until day 92 (Fig. 3). Urine collections were scheduled to avoid the 24-h period immediately after CDDP injections, because it was anticipated that the large saline load itself would produce a rise in urine volume.

The urines were centrifuged at 15 000 rpm for 15 min to remove cell debris, and the volumes were measured. They were then examined qualitatively for the presence of glucose, blood and ketone bodies and for changes in pH using Labstix (Ames Laboratories Ltd, Stoke Poges, UK).

Concentrations of sodium and potassium were measured in appropriate dilutions of urine by flame emission spectrophotometry, an internal standard of lithium being

Table 1. Activity of CDDP against the M5076 Sarcoma

Schedule[a] (days of injection)	Lethal dose values (mg 10 kg^{-1} day^{-1})		Antitumour activity (mg kg^{-1} day^{-1})	Therapeutic index
	LD_{10}	LD_{50}	ID_{90}	(LD_{10}/ID_{90})
0[b]	9.0	14.3	12.6	0.7
0, 4, 8, 12, 16	4.8	9.6	1.8	2.7
0 – 16	1.8	2.6	0.8	2.3

[a] Tumour volumes and deaths were recorded on day 24 from which the ID_{90}, LD_{10} and LD_{50} values were calculated

[b] The first day of injection was designated day 0. Tumour cells were implanted 24 h earlier

used. Calcium and magnesium were measured by atomic absorption spectrophotometry after appropriate dilution of the urine with a 0.1% solution of lanthanum chloride acidified with 50 m*M* hydrochloric acid. Creatinine was measured by the standard alkaline picrate procedure, osmolarity by freezing point depression, and protein by the method of Lowry et al. [16].

Enzymatic activity was assayed in urine which had been dialysed to remove low-molecular-weight inhibitors. Alanine aminopeptidase (AAP) activity was measured according to the method of Mondorf et al. [20] with L-alanine-4-nitroanilide hydrochloride as substrate. Lactate dehydrogenase (LDH) activity was measured at 37 °C by following the rate of change of absorption of NADH at 340 nm [14]. *N*-Acetyl-*β*-glucosaminidase (NAG) was measured using *p*-nitrophenyl-*N*-acetyl-*β*-glucosaminide as substrate [17]. All enzyme activities were measured as nanomoles of substrate changed per minute (milliunits) and expressed as total activity excreted in 24 h per milligram of creatinine.

Collection and analysis of plasma. Blood was obtained from each rat by cardiac puncture on days 6, 14, 22, 56, 71 and 92. The rats were anaesthetized with halothane and nitrous oxide, and blood was drawn into a heparinized syringe and centrifuged to obtain plasma. Calcium and magnesium were determined as described for urine. Blood urea nitrogen (BUN) was measured using a modified Urease-Berthelot reaction (Sigma Ltd., UK) and creatinine in an automated centrifugal analyser using a modified alkaline picrate procedure.

Cardiac punctures were always done immediately following a 24-h collection of urine. The plasma creatinine value (P_{cr}) and the urinary excretion of creatinine (urine concentration of creatinine times urine volume) during the previous 24-h period were used to calculate an approximate creatinine clearance (Cl_{cr}).

Histopathology. Kidneys and livers from rats were fixed in Bouin's solution and processed for light histology. Sections stained with Mayer's Haemalum and Eosin were examined by a histopathologist who had no knowledge of the drug treatments.

Statistical methods. In the antitumour assay and the myelotoxicity and nephrotoxicity studies, between-group comparisons were made by analysis of variance, followed by multiple comparisons among the various means. Statistical comparisons of the plasma enzyme data in the hepatotoxicity studies were done using the Mann Whitney U-test.

Results

Antitumour assays

The schedule dependency of CDDP against the M5076 sarcoma was first examined. Three treatment regimens – acute, intermittent and chronic – were compared (Table 1). The intermittent and chronic treatments induced similar responses, and both proved superior to the acute treatment when a comparison was made between the therapeutic indices of each treatment. On the basis of this result an intermittent treatment was used in the combination experiment.

Table 2. Dosage schedules used in the CDDP + NMF combination experiments

	CDDP	NMF	Schedule	No. dead[a]
	(mg kg^{-1} day^{-1})	(mg kg^{-1} day^{-1})	(days of injection)	Total
A	10	–	0, 4, 8, 12	5/10
B	5	–	0, 4, 8, 12	0/10
C	–	200	3 – 12	0/10
D	10	200	CDDP: 0, 4, 8, 12 NMF: 3 – 12	7/10
E	5	200	CDDP: 0, 4, 8, 12 NMF: 3 – 12	0/10
F	–	–	–	0/10

[a] Deaths were recorded on day 24

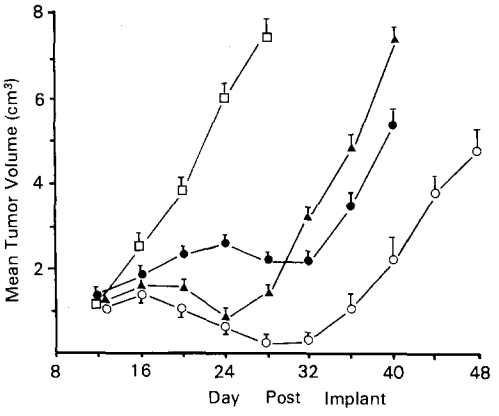

Fig. 1. Mean values (± SEM) for tumour volumes of mice treated with the M5076 sarcoma. Mice were treated as follows: 5 mg kg^{-1} CDDP (●); 200 mg kg^{-1} NMF (▲); 5 mg kg^{-1} CDDP and 200 mg kg^{-1} NMF (○); or saline (□)

We have previously shown that NMF, 200 mg kg^{-1} daily, administered according to a chronic schedule, was the optimal dose against the M5076 sarcoma [13], and this dose was therefore used in the present study.

In the combination experiment, mice bearing the M5076 sarcoma were treated with NMF alone, CDDP alone, or combinations of the two drugs according to the schedules described in Table 2. Since CDDP given in unit doses of 10 mg kg^{-1} (schedule A) killed 50% of the mice (Table 1) only the regimen involving unit doses of 5 mg kg^{-1}, with which no deaths occurred, was evaluated for antitumour effect, alone or in combination with NMF. The delays in the growth of mean tumour volumes in mice after treatment are illustrated in Fig. 1. The combination of CDDP + NMF (schedule E) proved superior to either NMF (schedule C) or CDDP (schedule B) alone. Thus, while the mean tumour volumes of mice treated with schedule C or B were not significantly different on day 40, those in mice treated with schedule E were significantly smaller ($P < 0.001$).

Leukopenia

Leukocyte counts for the treatments in Table 1 are illustrated in Fig. 2. Neither NMF alone (schedule C) nor 5 mg

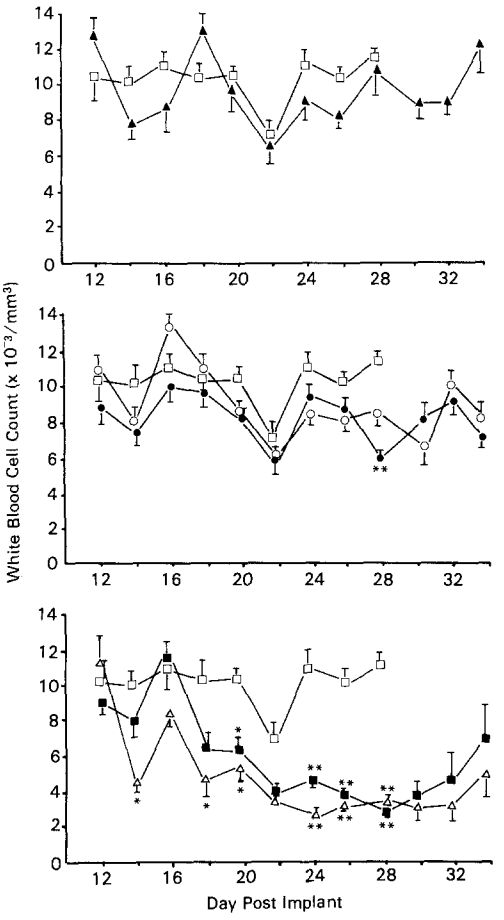

Fig. 2. Mean values (± SEM) for white blood cell counts in mice treated with saline (□), 200 mg kg^{-1} NMF (▲), 5 mg kg^{-1} CDDP (●), 5 mg kg^{-1} CDDP and 200 mg kg^{-1} NMF (○), 10 mg kg^{-1} CDDP (△) or 10 mg kg^{-1} CDDP and 200 mg kg^{-1} NMF (■). The significance of differences between the means of the drug-treated groups and the saline-treated controls is shown: *$P < 0.01$; ** $P < 0.001$

kg^{-1} CDDP, alone (schedule B) or in combination with NMF (schedule E), produced a significant reduction in the leukocyte count compared with values in control mice. CDDP, at approximately an LD_{50} dose in mice (10 mg kg^{-1}; schedule A), caused slight leukopenia, which was not augmented by the addition of NMF (schedule D). Thus, CDDP in combination with NMF was no more leukopenic than CDDP alone.

Hepatotoxicity

Plasma levels of three liver function marker enzymes (SDH, ALT and AST) were monitored after administration of the above combination (Table 3). SDH and ALT levels were slightly increased in the combination treatment (approximately 4-fold although this effect was small relative to the approximately 19-fold increase obtained when NMF was administered at a dose of 400 mg kg^{-1} day^{-1} over 10 days (Table 3).

Nephrotoxicity

The food intake of all rats injected with CDDP and NMF was significantly reduced and was associated with weight loss. Reduced food intake and altered metabolism can affect some of the parameters used in this study to monitor renal function. Consequently, the food intake of the other three groups of rats was matched to that of the CDDP+NMF group, so that the mean weight loss was similar in all groups. The rats treated with CDDP showed symptoms of lethargy and weakness, which was most marked in those that also received NMF; alopecia was also seen in the (CDDP+NMF) treated rats. Each of these toxic manifestations, anorexia, weight loss and alopecia, reversed after the drug administration was stopped. All rats regained a normal healthy appearance, and free feeding was resumed in all groups on day 42.

According to the Labstix test there were no changes in urinary pH at any time. Traces of glucose, ketones and blood were present in the urines of rats in the CDDP and CDDP+NMF groups from day 3 until the injections were stopped. One rat in the CDDP+NMF group passed large amounts of blood in its urine after two doses of CDDP. This animal was killed on day 8 because of its deteriorating physical condition. Histopathological examination revealed severe kidney damage characterized by numerous intratubular granular and hyaline casts in the cortex and medulla and many tubules throughout the cortex and outer medulla lined by basophilic epithelium. The latter were interpreted as attempts at regeneration following extensive necrosis. There were no significant changes in urinary protein excretion, except for a transient rise in the CDDP+NMF group on day 26. Rats were occasionally

Table 3. Plasma enzyme markers of drug-induced hepatotoxicity

Dosage schedules			Plasma enzyme levels								
CDDP	NMF	Schedule	SDH			ALT			AST		
(mg kg^{-1})	(mg kg^{-1})	(days of injection)	Day 3	Day 8	Day 13	Day 3	Day 8	Day 13	Day 3	Day 8	Day 13
5	–	0, 4, 8, 12	0.6	1.1	1.9	0.7	0.9	0.8	0.8	1.0	1.4
–	200	3 – 12	1.1	1.5*	1.5	1.5	1.1	1.5	1.3	0.7	1.4
5	200	CDDP: 0, 4, 8, 12	1.2	4.1*	2.7*	1.2	3.7*	1.2	1.1	1.7	1.2
–	400[a]	3 – 12	0.9	17.9*	19.2*	–	15.1*	15.6*	–	3.5*	2.8
saline injected controls		[b]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] Data from another study [12]
[b] Set at 1.0
* Significantly different from control values at $P < 0.05$ (Mann-Whitney U-test)

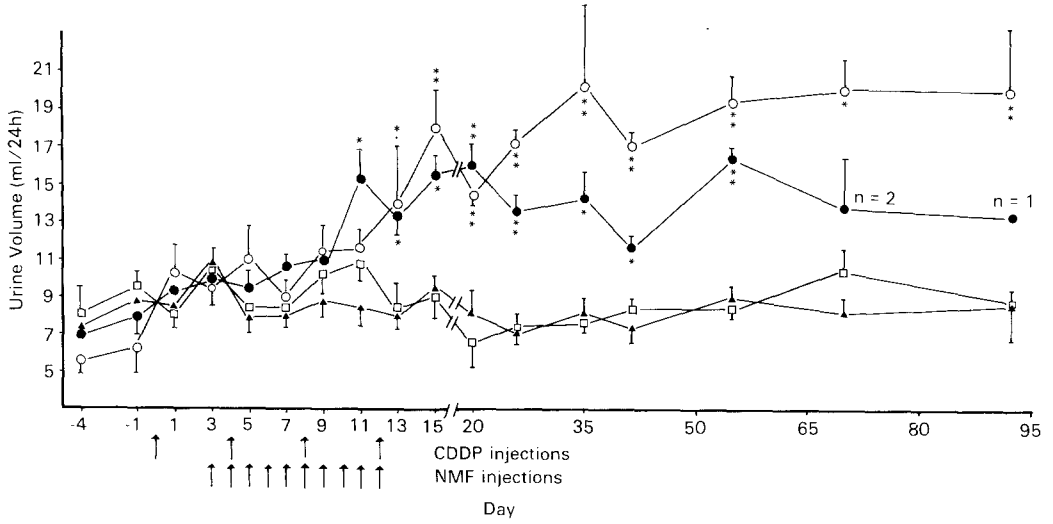

Fig. 3. Mean values (± SEM) for volumes of 24-h urines collected from rats ($n \geqslant 3$ except where indicated) treated on days denoted by the *arrows* with 2 mg kg^{-1} CDDP (●), 100 mg kg^{-1} NMF (▲), 2 mg kg^{-1} CDDP and 100 mg kg^{-1} NMF (○), or saline (□). The significance of differences between the means of the drug-treated groups and the saline-treated controls is shown: *$P < 0.01$; ** $P < 0.001$

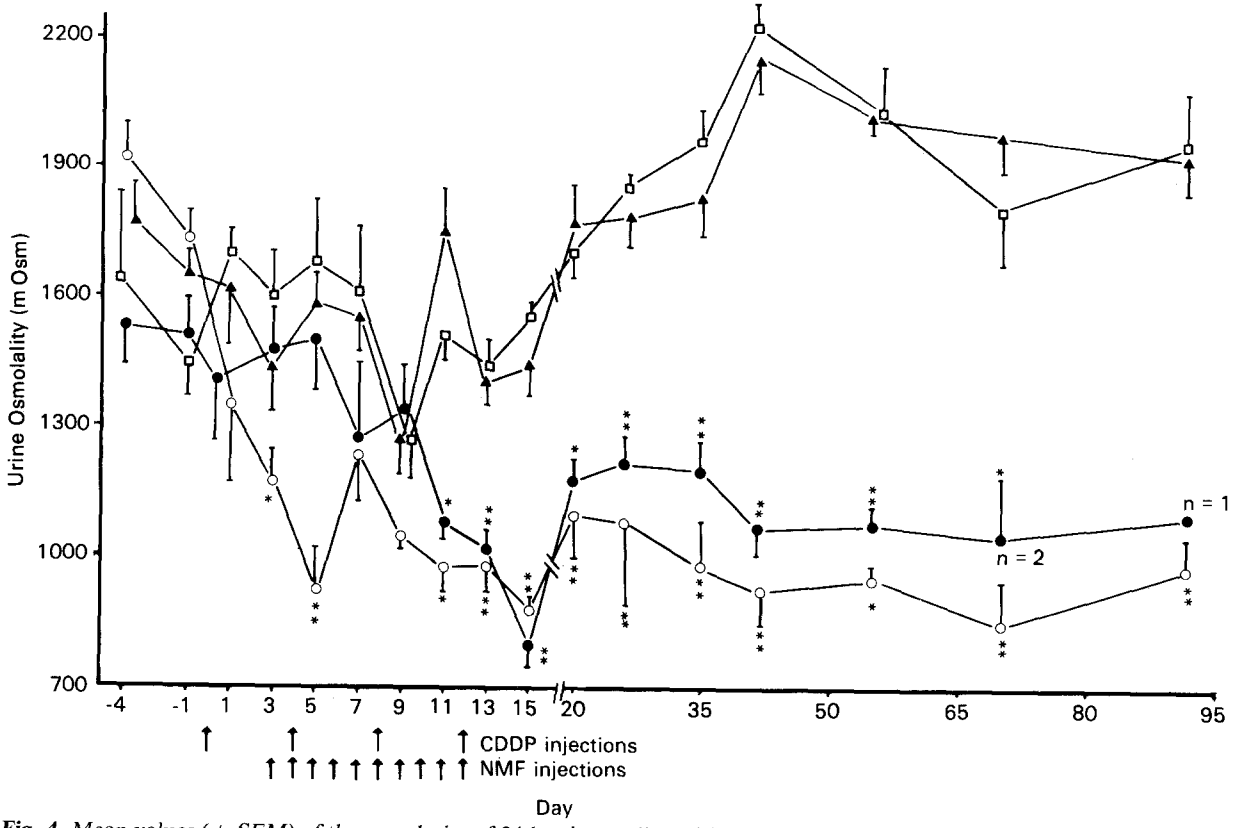

Fig. 4. Mean values (± SEM) of the osmolarity of 24-h urines collected from rats ($n \geqslant 3$ except where indicated) treated on days denoted by the *arrows* with 2 mg kg^{-1} CDDP (●), 100 mg kg^{-1} NMF (▲), 2 mg kg^{-1} CDDP and 100 mg kg^{-1} NMF (○), or saline (□). The significance of differences between the means of the drug-treated groups and the saline-treated controls is shown: *$P < 0.01$; ** $P < 0.001$

killed during the withdrawal of blood from the heart, but a minimum of three animals survived in each group until day 55.

Urine volume was significantly increased and urine osmolarity was significantly decreased in both the CDDP and CDDP+NMF groups, but not in the NMF group (Fig. 3 and 4). The decline in osmolarity preceded the rise in volume, but both the decreased osmolarity and the polyuria persisted for the duration of the study. The magnitude of the changes in urine volume and osmolarity were greater in the CDDP+NMF group than in the CDDP group, but the statistical significance of the changes compared with the controls was the same in both drug-treated groups. The gradual increase in urine volume between days 7 and 15 was followed by a sharp increase in water intake on day 15. Water intake was elevated for the rest of the study so that the animals remained in fluid balance.

Excretion of AAP and NAG in urine was slightly elevated in all groups, including the control group, during the dosing period. The excretion of both enzymes was charac-

terized by a series of transient peaks after each injection of CDDP, irrespective of whether or not the rats also received NMF. There seemed to be some cumulative effect, in that the greatest activity of AAP and NAG in the urine of the CDDP and CDDP+NMF groups occurred after the fourth dose of CDDP and was of almost identical magnitude – approximately three to four times the pretreatment values – in both the CDDP and CDDP+NMF groups (Fig. 5). The largest increase in activity of urinary enzymes was seen with LDH (Fig. 6) in the CDDP and CDDP+NMF groups (approximately 840% and 610% of pretreatment values, respectively). There was evidence of a biphasic effect, with the second and largest peak occurring after the last dose of CDDP. There was no difference between the activity of LDH in the urine of the CDDP and CDDP+NMF groups.

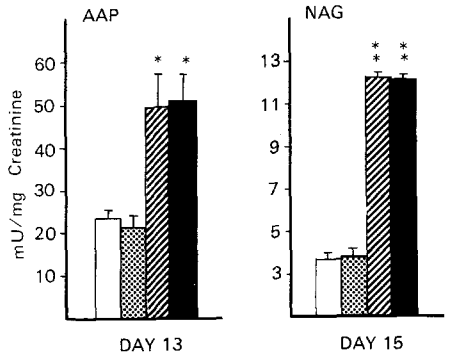

Fig. 5. Peak activities (mean ± SEM) of the enzymes AAP and NAG in 24-h urines collected from rats treated with CDDP (▨) or CDDP + NMF (■). Enzyme activities in the NMF group (▩) and the saline-treated controls (□) on the same days are shown for comparison. The significance of differences between the means of the drug-treated groups and the controls is shown: $*P<0.01$; $**P<0.001$

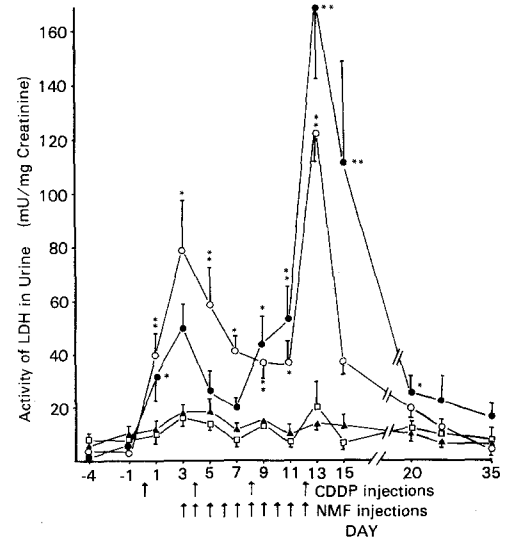

Fig. 6. Mean values (± SEM) for activity of LDH in 24-h urines collected from rats ($n \geq 3$) treated on days denoted by the *arrows* with 2 mg kg^{-1} CDDP (●), 100 mg kg^{-1} NMF (▲), 2 mg kg^{-1} CDDP and 100 mg kg^{-1} NMF (○), or saline (□). The significance of differences between the means of the drug-treated groups and the saline-treated controls is shown; $*P<0.01$; $**P<0.001$

After the dosing period the activities of all three enzymes in urine returned to pretreatment values: within 1 week of the last injection in the case of AAP and NAG and within 2 weeks in the case of LDH (Fig. 6). No enzyme assays were done after day 40. At no time did the activities of any of the enzymes in the urine of the NMF-treated rats differ from those of the controls.

There were occasional significant differences in sodium and potassium excretion between each of the drug-treated groups and the controls, mainly during the dosing period. Both increases and decreases in excretion were seen in the drug-treated rats with respect to the controls, so that no consistent pattern emerged. This was probably because excretion of sodium and potassium in all groups fluctuated from day to day, even to the extent that statistically significant within-group changes occurred in the control rats, apparently at random. Excretion of calcium and magnesium was transiently elevated between days 7 and 15 in the CDDP and CDDP+NMF groups. Whereas magnesium excretion did not differ between the CDDP and CDDP+NMF groups calcium excretion in the CDDP+NMF group was considerably greater than in the CDDP group (Fig. 7). There was also an ephemeral increase in the calcium excretion in the NMF group on day 11. Calcium excretion was the only parameter which showed a greater increase in the CDDP+NMF group than in the CDDP group compared with the controls. It was also the only parameter which was increased in the NMF group compared with the controls. None of these transient increases in urinary excretion of calcium and magnesium led to any significant decline in the concentrations of calcium and magnesium in plasma.

There was no difference between Cl_{cr} of any of the groups on any day except day 14, i.e., shortly after the last injections. On day 14 Cl_{cr} was decreased in all four groups of rats, presumably due to the reduced food intake. However, the greatest reduction occurred in the CDDP and CDDP+NMF groups, and their Cl_{cr} was significantly less than that in the controls (Fig. 8). BUN and P_{cr} were elevat-

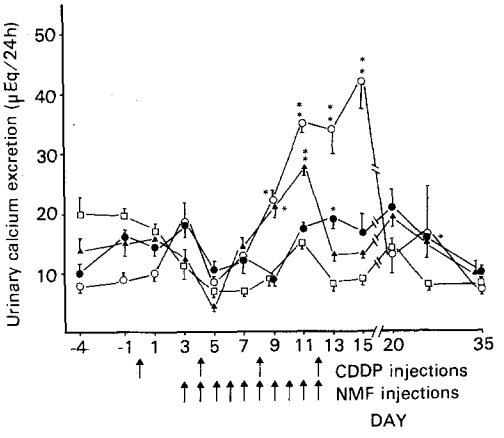

Fig. 7. Mean values (± SEM) for the excretion of calcium in 24-h urines collected from rats ($n \geq 3$) treated on days denoted by the *arrows* with 2 mg kg^{-1} CDDP (●), 100 mg kg^{-1} NMF (▲), 2 mg kg^{-1} CDDP and 100 mg kg^{-1} NMF (○), or saline (□). The significance of differences between the means of the drug-treated groups and the saline-treated controls is shown; $*P<0.01$; $**P<0.001$

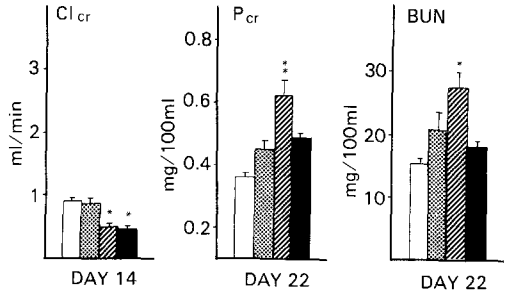

Fig. 8. Means values (± SEM) for creatinine clearance (Cl_{cr}) and plasma creatinine (P_{cr}) and blood urea nitrogen (BUN) on days when significant changes were seen. NMF group (▩), CDDP group (▨) and CDDP + NMF group (■). $*P < 0.01$; $**P < 0.001$ compared with the saline-treated controls (□)

ed in all four groups on day 14, and there were no differences between the groups. By day 22 BUN and P_{cr} had returned to normal in all groups except the CDDP group, where the values were significantly greater than in the controls (Fig. 8). The values for BUN and P_{cr} had returned to normal in the CDDP group by day 56.

The histopathological findings in the kidneys are summarized in Table 4. Kidneys from rats injected with saline or NMF showed only minimal background changes. Kidneys from animals killed after two doses of CDDP were either normal or showed only slight proximal tubular necrosis. Animals killed some time after the last injection exhibited severe chronic renal damage, mainly characterized by tubular dilatation and epithelial degeneration in the regions of the inner cortex and outer medulla, medullary hyaline casts and interstitial inflammatory cell infiltration. Although there was evidence of regeneration, particularly in animals killed some time after the end of CDDP administration, the degenerative lesions were still evident after 12 weeks. The renal damage in the animals injected with CDDP+NMF was similar to but generally less severe than that described for the CDDP group. This difference may be attributable to the longer survival times of some of the rats treated with CDDP+NMF. Only minor abnormalities were found in the sections of liver. There was no consistent histopathological evidence of hepatotoxicity in any group.

Table 4. Histopathological findings in the kidneys

	CDDP	CDDP/ NMF	NMF	Saline
Number examined	6	6	5	6
Number showing				
Tubular necrosis	1	1	0	0
Tubular casts	4	4	0	0
Tubular basophilia				
Minimal/slight	0	3	3	3
Moderate/marked	4	3	0	0
Tubular dilation	4	1	0	0
Interstitial inflammation	4	3	0	0
Interstitial fibrosis	4	1	0	0

Discussion

The aim of these studies was to determine whether or not the combination of CDDP+NMF might promise increased therapeutic benefit compared with either drug alone. Emphasis was placed on the toxicity of the compounds, and in particular on their major organ-directed toxicities, which are nephrotoxicity and hepatotoxicity, respectively.

The dosing schedule for NMF in mice, 200 mg kg^{-1} daily for 10 days, was previously shown to be optimal considering both antitumour response and lethal toxicity. An intermittent schedule of CDDP, four doses of 10 mg kg^{-1} at 4-day intervals, produced 50% mortality in mice, rising to 70% when this dose of CDDP was combined with 200 mg kg^{-1} NMF. Consequently, the highest dose of CDDP which could be evaluated for antitumour effect, either alone or in combination with NMF, was 5 mg kg^{-1}. With these dosage schedules, co-administration of CDDP+NMF resulted in an improved antitumour effect compared with CDDP or NMF alone. Under these conditions the leukopenia caused by CDDP was not influenced by the presence of NMF.

Hepatotoxicity was assessed by measurement of the plasma levels of the enzymes SDH, ALT and AST. A previous study of the hepatotoxicity of NMF in mice demonstrated that the elevation of these enzymes in plasma correlated well with the histopathological damage in the liver [27]. In BDF_1 mice it has been shown [12] that, given in a 10-day schedule, a dose of 200 mg kg^{-1} of NMF caused only a slight increase in plasma levels of SDH. This was confirmed in the present study. CDDP alone did not increase the plasma levels of any of the marker enzymes for liver damage, but CDDP+NMF caused a significant increase in SDH and ALT compared with NMF alone. However, the effect of the CDDP+NMF combination was small compared with that caused by a 400 mg kg^{-1} regimen of NMF (Table 3) [12]. This suggests that CDDP only slightly augmented the liver damage caused by NMF.

The Fischer rat was chosen for the nephrotoxicity studies because it has been shown to be sensitive to the nephrotoxic effects of drugs, including platinum compounds [25]. Changes in glomerular filtration rate (GFR) were monitored by measurement of Cl_{cr}, P_{cr} and BUN. CDDP may induce tubular damage, as indicated by the appearance in urine of enzymes of renal origin [2] and by increased excretion of electrolytes such as magnesium [5, 24], without any change in GFR. Thus, it was considered necessary in this study to monitor possible injury to tubular epithelia cells by measuring excretion of enzymes and to monitor tubular function by measuring electrolyte excretion together with urine volume and osmolarity.

Administration of CDDP caused changes in a variety of indices of renal function. There appeared to be a cumulative effect with successive doses of CDDP, most clearly evident in the urinary enzyme data. A similar cumulative increase in enzymuria has been observed in patients receiving therapy with CDDP [2]. Histopathological examination of the kidneys, which showed little damage after two doses of CDDP but extensive damage after four, also suggests that the effect at this low dose was cumulative. Although the absence of an increase in protein excretion suggests that there was no acute episode of tubular necrosis, nevertheless the tubular lesions were apparently severe

enough to result in a depression of Cl_{cr} and a rise in P_{cr} and BUN. All the changes reversed after the CDDP administration was stopped, with the exception of the polyuria and decreased osmolarity of urine, which persisted for the 3 months' duration of the study. CDDP therapy in patients has been reported to result in subclinical, permanent renal injury, as manifest in a decrease in GFR [6] or a raised excretion of enzymes [10]. The results of this study suggest that tubular dysfunction caused by CDDP may persist even when GFR has returned to normal. The histopathological studies provided further evidence for the presence of a chronic renal lesion.

NMF, 100 mg kg^{-1} daily for 10 days, had no effect on any of the measured indices of renal function with the exception of calcium excretion, which was transiently increased. The cause or significance of this was not investigated, although it was shown recently that NMF inhibits mitochondrial, but not microsomal, uptake of calcium in these fractions prepared from liver cells [26]. CDDP, which is known to cause electrolyte disturbances, produced a transient increase in calcium and magnesium excretion. The increased excretion of calcium was greater in the (CDDP + NMF)-treated rats compared with the CDDP-treated rats. No other index of renal disturbance was greater in the CDDP + NMF group than the CDDP group alone. The effects on calcium excretion were transient, and the significance of this observation is speculative, since although hypocalcaemia has been associated with CDDP therapy [22] it has been thought to occur secondary to renal wasting of magnesium [1]. The results of the functional studies supported by histopathological evidence suggest that NMF was not nephrotoxic at the dosage schedule used in this study and did not augment the tubular damage caused by CDDP.

The only rat to die during the period of drug administration was in the CDDP + NMF group. Furthermore, the animals in this group developed reversible alopecia and appeared weaker and more lethargic than the animals in either of the NMF or CDDP groups. These effects rapidly reversed after drug administration was stopped. In clinical use, NMF commonly caused a general feeling of tiredness and sleepiness [18] but these effects seemed minor in comparison to the hepatotoxicity and gastrointestinal toxicity of the drug.

The mechanism of the antitumour action of NMF remains unsolved, although it has been shown to induce differentiation in various tumor cell lines in vitro [4] and also to possess radiosensitization properties [9]. CDDP is known to have radiomimetic properties and to induce lesions in DNA. Pretreatment of cultured human colon cells with NMF can result in up to a 10-fold enhancement of the cytotoxic effect of CDDP [8]. On the basis of this finding it has been suggested that the efficacy of the combination of CDDP + NMF should be assessed in clinical studies. In this study CDDP and NMF were shown to have an additive antitumour effect against the M5076 sarcoma compared with either drug alone, whereas in mice and rats there was no significant augmentation of the individual toxicities of the compounds. Thus, the results of the present investigation provide complementary evidence to support this suggestion that clinical evaluation of the combination is merited.

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