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Original Paper

Effects of Cisplatin and Amphotericin B on DNA Adduct Formation and Toxicity in Malignant Glioma and Normal Tissues in Rat

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In an attempt to modify the cytotoxicity of cisplatin, amphotericin B (AmB) was given as pretreatment to BDIX rats with intracerebral BT4C glioma implants. Ten animals given AmB 5 mg/kg i.p. followed by cisplatin 5 mg/kg i.p. displayed massive haematuria within 24 h after treatment and died a few days later. The antitumoral effect could not, therefore, be evaluated. Histopathological examination of the kidneys showed extensive tubular necrosis. No signs of apoptotic cell death were found using *in situ* end labelling with biotin-labelled nucleotides or with DNA integrity analysis in agarose gel electrophoresis. An immunohistochemical method for analysis of cisplatin–DNA adducts was used to elucidate the distribution of cisplatin in brain tumour, normal brain and kidney. Addition of AmB to cisplatin caused increased adduct formation in kidneys, particularly in tubular cells. It seems plausible that the nephrotoxicity, at least in part, was mediated by increased levels of cisplatin–DNA adducts. Pretreatment with AmB did not have any obvious effect on the formation of adducts in the cerebral cortex. The adduct levels in the tumours from animals pretreated with AmB were not significantly increased compared with those treated with cisplatin only. Thus, addition of AmB to cisplatin caused excessive nephrotoxicity suggesting a decrease in the therapeutic ratio of cisplatin. © **1997 Published by Elsevier Science Ltd. All rights reserved.**

Key words: glioma, chemotherapy, cisplatin, amphotericin B, DNA adducts, renal toxicity

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INTRODUCTION

CISPLATIN (CIS-DIAMMINEDICHLOROPLATINUM (II)) is one of the most potent anticancer drugs in the treatment of a variety of solid tumours. It has also been applied against malignant brain tumours, but alone it has only modest activity against malignant gliomas [1]. The concentrations of cisplatin have been shown to be low in normal brain tissue whereas the platinum concentrations in brain tumours are higher and comparable with the levels in extracerebral tumours [1]. The antitumour activity of cisplatin is thought to be mediated through binding to DNA and formation of adducts and cross-links that lead to inhibition of DNA synthesis and transcription. It has also been demonstrated that cisplatin induces apoptosis (programmed cell death) *in vitro* [2–4] and studies using the DNA fragmentation assay on human glioblastoma cell lines have demonstrated that synergism between cisplatin and nifedipine results in apoptosis [5].

With the purpose of enhancing the antitumoral effect of cisplatin, a number of agents have been investigated [6], of which amphotericin B (AmB) is one. AmB is a macrolide polyene antibiotic clinically used in the treatment of disseminated fungal infections. The activity of AmB is related to its ability to react with membrane sterols, causing alterations in membrane integrity and permeability. It binds preferentially to ergosterol, a fungal membrane sterol, but also binds to cholesterol, abundant in mammalian cell membranes. AmB has been shown to increase the intracellular accumulation of cisplatin and other anticancer agents in tumour cells [7–11], and thereby augmenting the cytotoxic effects. The effect of AmB on the formation of cisplatin–DNA adducts in tumours and normal tissues *in vivo* is yet unknown.

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The main objective of the present study was to investigate whether AmB could be utilised to increase the therapeutic ratio of cisplatin in the treatment of malignant brain tumours and to investigate in a rat glioma model if the addition of AmB to cisplatin increases the formation of cisplatin–DNA adducts and whether this is reflected in enhanced apoptosis.

MATERIALS AND METHODS

Animal model

A rat glioma model with inbred BDIX rats was set up and the nitrosurea-induced rat glioma cell line BT4C was used. This BT4C autologous tumour (kindly provided by Bjerkvig, Bergen, Norway) was originally serially transplanted in BDIX rats from which it was derived and it has been characterised as a pleiomorphic mixed glioma [12]. The cells were kept at −70°C until use and they were grown as a monolayer for one week before implantation. Cells growing in log phase were harvested and trypsinised before being centrifuged, and the pellet was diluted in MEM (minimal essential medium) with 5% BDIX rat serum to a concentration of 20 000 cells/5 µl. Rats aged 2 months were anaesthetised with i.p. administration of 1.8 ml/kg of a 1:1 mixture of Hypnorm® (fluanisonum 10 mg/ml and fentanylum 0.2 mg/ml) and Dormicum® (midazolam 5 mg/ml). The mean weight of the rats was 268 grams (range 190–383 g). 20 000 viable cells were transplanted under stereotactic conditions 3.5 mm to the right of the bregma at 4.5 mm depth in the caudate nucleus using a microsyringe (22S Ga needle: Unimetrics, Shorewood, IL, U.S.A.) allowing 5 min for injection and withdrawal of the needle to prevent cellular reflux and extracerebral spread of tumour cells. The drill-hole was closed with bone wax. To ensure cell viability, the cell suspension was continuously controlled by staining with trypan blue. After implantation, the rats were fed *ad libitum*. The studies were approved by the Ethics Committee at Umeå University.

Effect on tumour growth

In the efficacy study, rats with tumours 12 days after implantation were assigned to one of three treatment alternatives; AmB (Fungizone®, Bristol-Myers Squibb) 5 mg/kg i.p., cisplatin (Platinol®, Bristol-Myers Squibb) 5 mg/kg i.p., or AmB 5 mg/kg + cisplatin 5 mg/kg with 4 h interval between drugs. Ten animals were given the combined treatment, there were five animals in the other two treatment groups and another five untreated rats served as controls. Tumour volumes were measured 12 days after treatment when the rats were sacrificed and the brains were taken out and fixed in chilled ethanol. The width and height of the tumour was measured at the largest coronal section using calipers. The tumour volume was calculated using the formula for an ellipsoid ($r_1 \times r_2 \times r_3 \times \pi \times 4/3$) where the radius in the sagittal plane was approximated to be the same as the radius in the coronal plane (width).

Histopathology

For histopathological examination of the kidneys, 6 rats were divided into groups receiving treatment as above with AmB, cisplatin or a combination of the drugs, 2 animals in each treatment group. Two rats served as untreated controls. All animals were sacrificed after 48 h, the kidneys were fixed in 4% formaldehyde and processed for routine staining with haematoxylin and eosin.

Cisplatin–DNA adduct analysis

Twenty days after tumour implantation, 24 rats were divided into two groups and treated with either cisplatin 5 mg/kg i.p. or AmB 5 mg/kg i.p. + cisplatin 5 mg/kg i.p. with an interval of 4 h between drugs. The rats were sacrificed at 1, 6 and 24 h after cisplatin administration. Thus, there were four animals at each time point per treatment. Tumour, normal brain tissue from the contralateral hemisphere and kidneys were collected and frozen in liquid nitrogen. Four untreated rats served as controls. Multiple 10 µm cryostate sections from the frozen specimens were mounted on poly-L-lysine coated slides and stored at −70°C until immunostaining for cisplatin–DNA adducts.

Cisplatin–DNA adducts were visualised immunohistochemically by using the antiserum NKI-A59, which is a polyclonal antiserum that recognises bifunctional intrastrand cross-links [13]. The immunostaining protocol of Terheggen and coworkers [14] was used with some modifications as previously described [15]. The general outlines were as follows. A maximum of 19 slides could be stained in each batch. Thus, each organ type had to be divided into two separate staining batches, containing equal numbers of samples from the two treatment groups and the different time points after drug administration. Each batch also contained a control slide with kidney samples from one cisplatin treated and one control rat to allow for correction between staining batches. The slides were treated with H_2O_2 and NaOH before incubation with anti-cisplatin–DNA rabbit antiserum, NKI-A59 (a gift from Drs den Engelse and Blommaert, Netherlands Cancer Institute, Amsterdam), diluted 1:1500 in PBS, with 10% fetal calf serum and 5% calf thymus DNA. Slides were then sequentially incubated with goat-antirabbit immunoglobulin (Dakopatts, Copenhagen, Denmark), diluted 1:600 and peroxidase antiperoxidase complex (American Qualex, La Mirada, CA, U.S.A.) diluted 1:3000. The goat-antirabbit immunoglobulin and the peroxidase antiperoxidase steps were repeated once. A brown staining reaction was developed with diaminobenzidine and the slides were finally counterstained with methyl green.

Quantitation of the cisplatin–DNA adducts was performed with the computer-assisted image analyser CAS200 (Cell Analysis System, Elmhurst, IL, U.S.A.), using a two-colour mask image technique [16]. The quantitative nuclear antigen (QNA) software package was utilised. The image analysis procedure has been thoroughly described and evaluated in a previous report [15]. Since each tissue type has its specific staining pattern with adducts in different histological structures, the topographic areas of interest were decided upon for each organ type. On each slide 10–30 representative 40× fields, with a minimum of tissue folds and other artefacts, were measured. A total of 200–2000 cells per slide were thus analysed. For each field, the CAS computer calculates the 'positive area' which represents the percentage of positive nuclear area (PNA). The PNA values of each single field are merged to produce mean values for each slide. To adjust for non-specific nuclear staining, the PNA values of the slides from the untreated control animals were subtracted from the remaining slides of that staining batch.

Staining levels can vary considerably between batches [15]. To correct for this interbatch variation, PNA values of the control slides were used to calculate correction factors according to which all measurements were adjusted.

Apoptosis

Twenty days after tumour implantation, three groups of three rats were treated with AmB 5 mg/kg i.p., cisplatin 5 mg/kg i.p. or AmB + cisplatin with a 4 h interval between the drugs. The animals were sacrificed 5 h after cisplatin administration and tumour tissue, normal brain and kidney were frozen in liquid nitrogen or fixed and embedded in paraffin. Two non-treated animals served as controls. Two different techniques were used to study apoptosis. Both of them demonstrate DNA fragmentation, which is an important characterisic of the apoptotic process.

In situ *end labelling (ISEL)* is a method for visualisation of apoptotic cells in terms of DNA strand-breaks. The protocol of Wijsman and coworkers [17] was used. 3–4 μm thick sections were cut from the paraffin-embedded specimens according to routine histology procedures. After deparaffinisation and rehydration, the sections were heated twice in SSC (sodium chloride 17.5 g, sodium citrate 8.8 g/l water, pH 7.0; Merck Darmstadt, Germany) at 80°C for 20 min and subsequently washed thoroughly in distilled water. To enable enzymatic incorporation of nucleotides, the sections were digested in 0.5% pepsin in hydrochloric acid (pH 2) for 15 min with gentle shaking in a 37°C water bath. The digestion was stopped by washing several times in tap water and then washed in buffer for 5 min. After drying, the sections were incubated for 1 h at 15°C with buffer containing 0.01 mM dATP, dGTP, dCTP and 0.01 mM biotin dUTP (Boehringer Mannheim, Germany) along with 4 U/ml DNA polymerase 1 (Sigma, U.S.A.). Endogenous peroxidase was blocked for 5 min in PBS with 0.1% hydrogen peroxide, and the sections were then washed twice in PBS (0.1% hydrogen peroxide). The sections were incubated with avidin dissolved in PBS with 1% BSA (bovine serum albumin) and 0.5% Tween 20 (Boehringer Mannheim) for 30 min at room temperature before developing with diaminobenzidine. In negative controls DNA polymerase was excluded from the nucleotide polymerase mix. Normal rat prostate, 3 days after castration, was used as a positive control since under these conditions a significant number of the epithelial cells display characteristic features of apoptotic cells [18]. The number of ISEL-positive cells was quantified in the light microscope.

Analysis of DNA integrity with electrophoresis. The method demonstrates DNA fragmentation and has recently been described in detail [19]. The frozen tissues were ground with a homogeniser. Tissue fragments were suspended in digestion buffer (100 mM sodium chloride, 10 mM Tris HCl pH 8, 25 mM EDTA pH 8, 0.5% sodium dodecyl sulphate and 0.1 mg/ml proteinase K) and incubated at 50°C for 12 h and each sample was treated with DNAse free RNAse (0.5 mg/ml) for 1 h. The samples were heated to 70°C. Loading buffer (0.25% Orange G, 30 mM EDTA, 15% Ficoll) was added to each sample at a 1:2 ratio before loading into the 1.5% agarose gel containing 0.1 μg/ml ethidium bromide.

Electrophoresis was carried out in 0.1 M TBE (0.045 M Tris–borate, 0.001 M EDTA) at 21V for 14–16 h and viewed by transillumination with ultraviolet light. (Hind III standard was used as molecular size standard. All chemicals were from Sigma, U.S.A.)

Statistics

For comparison of PNA values of the cisplatin–DNA adduct analysis in the two treatment groups, a two-sided analysis of variance was performed with time and treatment as factors. Differences in PNA values between the whole tumours and the tumour periphery were analysed by means of paired comparisons and Student's *t*-test. Differences in tumour volumes were analysed by Mann–Whitney test.

RESULTS

Tumour and tissue reaction

In animals treated with cisplatin, there was an average 40% reduction in tumour volume compared with controls and in those given only AmB a 25% reduction was seen 12 days after treatment (Table 1), although the differences were non-significant. All rats treated with the combination AmB + cisplatin displayed massive haematuria within 24 h after injection and were all sacrificed 3 days after drug administration when the first deaths had occurred. Thus, the tumoricidal effect of the combined treatment could not be evaluated. Haematuria was not seen among animals given AmB or cisplatin alone. Sections from kidneys of animals treated with the combination AmB + cisplatin revealed extensive tubular necrosis (Figure 1). This was not seen in kidneys of rats given either drug alone.

Cisplatin–DNA adducts

Although the tumours were relatively homogenously stained for adducts, there was a general tendency for stronger staining in the periphery than in the central part of the tumours. Therefore, each tumour was subjected to two measurements; the whole tumour and the peripheral parts were analysed separately. Some tumours contained areas of necrosis in which adducts were not measured. The PNA values were significantly higher ($P < 0.001$) in the periphery than in the whole tumour slices. There was no significant difference in adduct levels in the tumours from animals pretreated with AmB compared with those treated with cisplatin only (Figure 2a and b). There was a gradual increase in tumour cell adducts during the study period of 24 h in both treatment groups ($P < 0.05$).

In the normal brain, contralateral to the tumour, staining was seen in neuronal as well as in glial cells. The way the sections were prepared did not allow a standardised analysis of the deeper structures so only the cortical parts were measured. Pretreatment with AmB did not have any obvious effect on the formation of adducts in the cerebral cortex (Figure 2c). There were high levels of nuclear background staining, e.g. staining in brains from untreated ani-

Table 1. Mean volume and range of brain tumours in BDIX rats at day 24 after implantation

Treatment (*n*)	Mean volume (mm^3)	Range	
Controls (5)	223	145–352	
Cisplatin (5)	131	55–195	($P = 0.17$)
Amphotericin B (5)	167	63–272	($P = 0.60$)

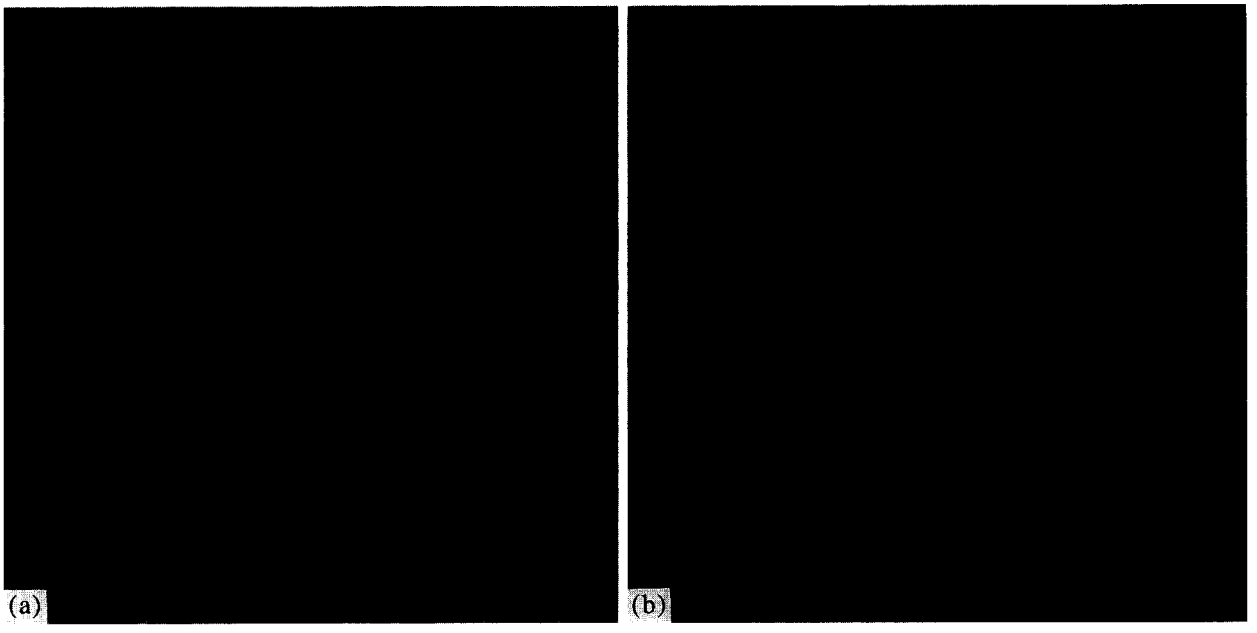

Figure 1. Massive tubular necrosis in rat given AmB 5 mg/kg + cisplatin 5 mg/kg (a) compared to normal renal cortex (b). Magnification 100×.

mals and the interindividual variations within each time point and treatment group were large.

In the kidneys, three regions could be identified, the outer and inner cortex and the medulla. The formation of cisplatin–DNA adducts was more pronounced in the cortex than in the medulla, especially in some tubular structures. To allow comparisons between kidney slides containing different proportions of the three topographic regions, all measurements were made in the outer cortex. There were significantly higher PNA values ($P < 0.001$) in the kidneys from animals pretreated with AmB (Figure 2d).

Apoptosis

In untreated control animals, only occasional single ISEL positive cells were seen in tumour, normal brain and kidney. There was no obvious increase in ISEL positive cells in either tissue in specimens from rats treated with Amb, cisplatin or AmB + cisplatin 5 h after treatment. DNA fragmentation typical of apoptosis could not be detected with DNA electrophoresis in any of the specimens.

DISCUSSION

In the present study, we found that 5 mg/kg of AmB given 4 h prior to 5 mg/kg of cisplatin in an *in situ* rat glioma model resulted in lethal nephrotoxicity and significantly increased levels of cisplatin–DNA adducts in kidneys, but not in tumours. It appeared that the enhanced kidney damage was not mediated by apoptosis.

The nephrotoxicity of cisplatin is primarily of tubular origin [20], but the precise cellular mechanism is not completely understood. Cisplatin nephrotoxicity has been related to structural changes in nuclear DNA of tubular cells [21] and high levels of cisplatin–DNA adducts are also formed in kidneys [22–24]. These findings suggest that the renal damage is mediated by drug interaction with DNA. Other mechanisms such as interference with mitochondrial function have also been reported [25]. In the present study, extensive nephrotoxicity after combined treatment with AmB and cisplatin was accompanied by increased levels of cisplatin–DNA levels in kidneys, particularly in tubular cells. This observation indicates that adduct formation in kidneys is related to nephrotoxicity. AmB in itself can also cause tubular kidney damage [26], but this effect is usually mild and reversible, and in the present study no significant nephrotoxicity was observed after treatment with AmB alone. However, it cannot be ruled out that the direct toxic effect of AmB may have contributed to the total nephrotoxicity observed after treatment with AmB and cisplatin. With the intention to decrease the toxicity, a liposomal formulation of AmB (AmBisome) has recently been introduced. AmBisome has proven less nephrotoxic than AmB in the clinical setting [27]. In a pilot study at our laboratory, the combination of AmBisome and cisplatin was investigated with the same *in vivo* model, but this combination seemed to be equally nephrotoxic as treatment with parental AmB and cisplatin (data not shown). Hence, our results indicate that the enhanced nephrotoxicity, at least in part, was induced by an increased cellular uptake of cisplatin and an increased formation of cisplatin–DNA adducts caused by pretreatment with AmB.

The cisplatin–DNA adduct analyses were conducted by an immunohistochemical approach. Quantitative analysis with this method may be performed with slides of the same tissue type, whereas quantitative comparisons including different tissue types should be avoided, due to differences in staining pattern [15]. For this reason, no quantitative comparisons of the adduct levels could be made between tumour and normal brain. However, a subjective microscopical evaluation definitely gave the impression that there were more adducts in tumour than in the opposite hemisphere of the normal brain. This is in agreement with previous studies showing higher platinum concentrations in brain tumours than in the normal brain [28]. They also found a decreasing gradient of platinum levels with increasing distance from the tumour. One advantage with the immunohistochemical method is that it allows investigations

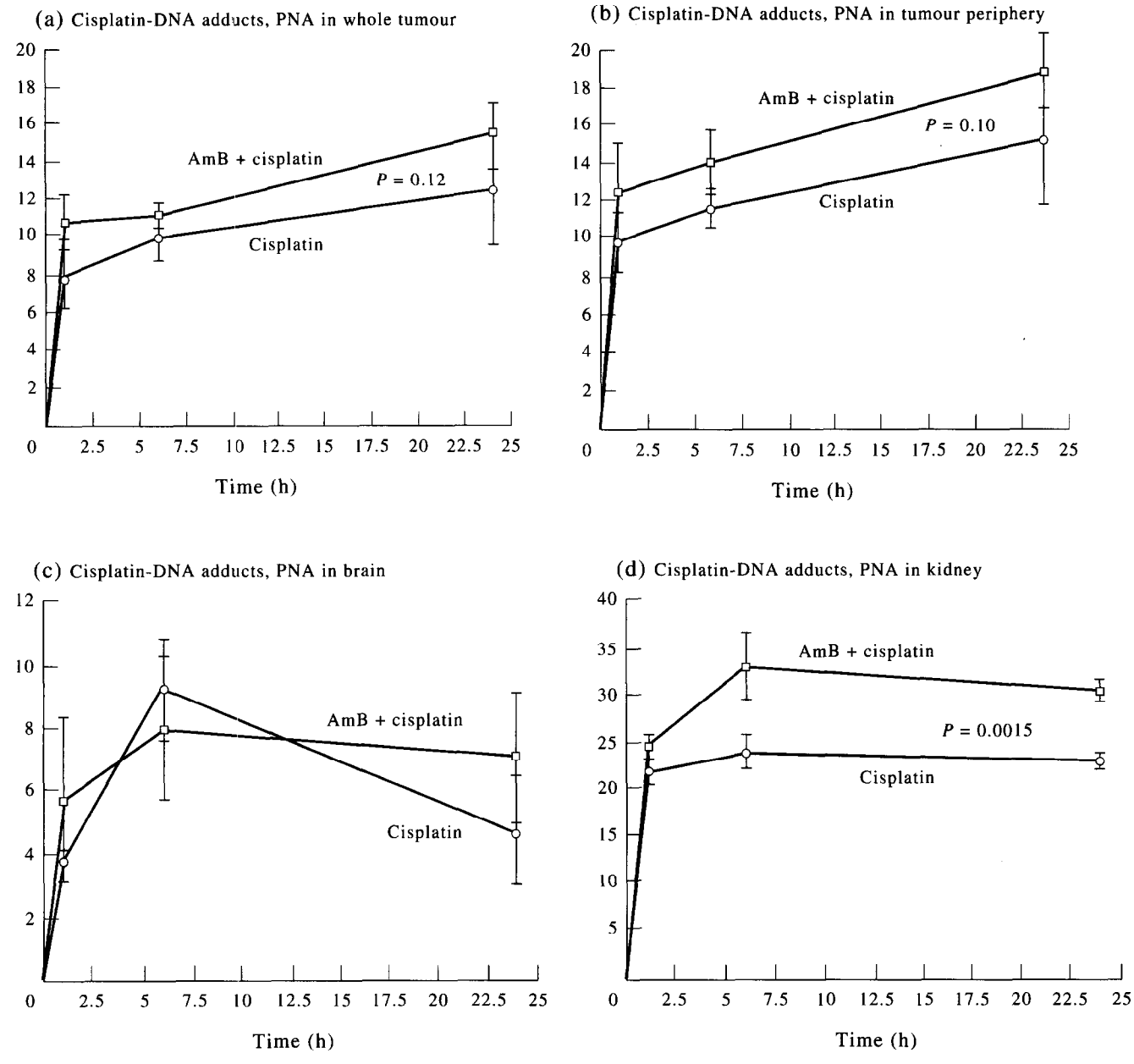

Figure 2. Cisplatin–DNA adducts in whole tumour (a), tumour periphery (b), normal brain (c), and kidney cortex (d) after i.p. treatment of glioma-bearing rats with cisplatin 5 mg/kg or AmB 5 mg/kg + cisplatin 5 mg/kg. Points indicate mean PNA of animals, ±SE.

on the distribution of adducts within tissues. In the present study, this topographic potential was utilised in the tumour samples. They showed significantly higher levels of cisplatin–DNA adducts in the periphery than in the central parts of the tumours, possibly due to differences in vascularisation.

The mechanism by which AmB potentiates the cytotoxicity of cisplatin is thought to be an effect on the cell membrane resulting in increasing uptake of cisplatin. AmB has been shown to increase the tumour cell concentration of platinum *in vitro* and *in vivo* [11] and the number of interstrand cross-links *in vitro* [10]. However, in the present study, no significant increase in cisplatin–DNA adducts in tumour cells was seen by combining cisplatin with AmB and the antitumoral effect of the AmB and cisplatin combination could not be evaluated, since the animals were sacrificed prematurely due to the renal toxicity.

To elucidate further the mechanism for AmB's potentiation of cisplatin nephrotoxicity, signs of apoptosis were also investigated by means of two different methods for detection of DNA degradation, which is an important feature in apoptotic cell death. Cisplatin has previously been shown to cause apoptosis [2] and it has been demonstrated that cisplatin and nifedipine (a calcium-channel blocker) in combination can induce apoptosis in human glioblastoma cells at a concentration of cisplatin which when tested alone did not induce apoptosis [5]. The apoptotic response is known to be dynamic in time and, using our rat tumour model, it has been shown that after treatment with estramustine apoptotic DNA degradation is evident 0.5–8 h after

treatment, but barely detectable after 24 h [29]. Other studies [2] show that loss of membrane integrity in Chinese ovary cell lines occur 12–24 h after cisplatin-induced DNA digestion. In the present study, AmB + cisplatin resulted in massive haematuria within 24 h and we assume that if apoptotic cell death had been part of the mechanism some evidence of apoptotic DNA degradation would have been seen 5 h after treatment. This was not the case in our study. Thus, the results indicate that the observed renal toxicity does not include an apoptotic cell death. The histopathological examination revealed large areas of tubular necrosis in kidneys from animals treated with the AmB + cisplatin combination.

The lethal nephrotoxicity noted in the present study is in contrast with a study by Kojima and coworkers [11] who found a prolonged survival time without enhanced toxicity in tumour-bearing nude mice treated with cisplatin 2 mg/kg + AmB 2 mg/kg. Differences in doses, timing and species may contribute to this discrepancy. The difference in species could be of some importance, but the doses used have been tolerated in other studies with cisplatin or AmB [30–33]. AmB 5 mg/kg is well tolerated in our rat model, but further studies indicate that cisplatin 5 mg/kg is close to the maximum tolerated dose. Kojima and coworkers gave AmB and cisplatin simultaneously and the difference in schedule could be of importance. It has been demonstrated that the interactive effect of AmB on chemosensitisation is dependent on sufficient exposure time before the chemotherapeutic agent is administered [34].

In conclusion, by using an *in vivo* rat glioma model, it has been demonstrated that analysis of cisplatin–DNA adducts in tumours and normal tissues can be a valuable tool for assessing the mechanisms of interaction between cisplatin and chemomodulating agents. Addition of AmB and cisplatin caused excessive nephrotoxicity and an increased adduct formation in the renal cortex but no significant increase in tumour tissue. Thus, it is suggested that AmB in this animal model decreased the therapeutic ratio of cisplatin.

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