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Metoclopramide as a modulator of cisplatin: effects on pharmacokinetics and cisplatin–DNA adducts in tumor and normal tissue

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The antiemetic drug metoclopramide (MCA) has previously been shown to cause DNA damage, to inhibit DNA repair and to enhance the effect of the chemotherapeutic agent cisplatin. Cisplatin acts by binding to DNA and thus forming cisplatin–DNA adducts. The present study was designed to investigate whether MCA affects the pharmacokinetics of cisplatin and the levels of cisplatin–DNA adducts in tumor and kidney. The effect on kidney is of special interest since cisplatin is highly nephrotoxic. Nude mice with xenografted squamous cell carcinoma where injected with cisplatin 5 mg/kg i.p. alone or in combination with MCA 2 mg/kg i.p. MCA was given 8 h after cisplatin. Total platinum was measured in serum and cisplatin–DNA adducts were analyzed in tumor and kidney with quantitative immunohistochemistry at 1, 9 and 24 h after cisplatin administration. The efficacy after treatment with cisplatin, MCA or cisplatin + MCA was studied in terms of tumor size measurements during 3 weeks following treatment and our previous observation that MCA enhances the cisplatin cytotoxicity was confirmed. The addition of MCA to cisplatin resulted in a slight increase in serum-platinum concentrations at 9 h and in increased levels of adducts in tumors at 24 h. There was a tendency, however, not statistically significant, for increased adducts also in kidney. Thus, our findings may indicate that the sensitization of MCA on the cytotoxicity of cisplatin is mediated by increased formation, maybe accompanied by inhibited repair, of cisplatin–DNA adducts.

Key words: Adduct, cisplatin, DNA, metoclopramide.

Introduction

Cisplatin (*cis*-diammine-dichloroplatinum(II), CDDP) is a potent chemotherapeutic agent with activity against a variety of tumors, e.g. testicular, ovarian and head/neck. Cisplatin also has substantial side-effects of which nephrotoxicity usually is dose limiting. The mode of chemotherapeutic action of cisplatin is presumed to be primarily binding to DNA and thus forming cisplatin–DNA adducts, leading to inhibition of DNA synthesis.

With the purpose of enhancing the antitumoral effect and increasing the therapeutic index of cisplatin, a number of agents have been investigated,[1] of which metoclopramide (MCA) is one. MCA is clinically used as an antiemetic drug, but animal studies have demonstrated that MCA also can act as a sensitizer of ionizing radiation[2] and cisplatin.[3,4] The mechanism for this potentiation is thought to be an increase of DNA damage as well as inhibition of DNA repair.[5,6] Whether the addition of MCA affects the levels of cisplatin–DNA adducts or the pharmacokinetics of cisplatin is not known.

The aim of this study was to further elucidate the mechanisms for MCA's potentiation of the cytotoxicity of cisplatin, by investigating the pharmacokinetics in terms of total platinum in plasma, and the cisplatin–DNA adducts in xenografted tumors and kidneys from nude mice treated with single doses of cisplatin, with or without addition of MCA.

Materials and methods

Animals

Five- to 8-week-old, male and female, BALB/c nude mice were used. Only animals showing progressive weight gain and weighing more than 18 g before treatment were used.

Tumor line

A poorly differentiated squamous cell carcinoma originating from a patient with carcinoma of the nasal cavity was used in its 144th passage. This tumor line has previously been used in our studies

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demonstrating MCA sensitization of cisplatin treatment.[3,4] The tumors were serially transferred by s.c. inoculation of 2 × 2 × 2 mm pieces on both flanks of the animal.

Design of efficacy study

A total of 40 mice were divided equally into four treatment groups: (1) physiological saline, NaCl, (2) cisplatin, (3) MCA or (4) cisplatin + MCA. Doses and time intervals are given below. Tumor volumes were recorded before treatment and then three times weekly for the 3 weeks following treatment.

Design of pharmacokinetic study

There were a total of 31 animals included and divided into six treatment and sampling groups according to Figure 1. An additional 17 animals were excluded from the analysis, six of them because of insufficient amount of blood for serum platinum analysis. Eleven animals were excluded since their serum platinum levels were clearly lower than expected, six of them had platinum concentrations below the detection limit (50 ng/ml) and in the remaining three the platinum level was more than 2 SD below the expected levels based on previous experiments,[7] possibly due to mis-injection of the drug.

Treatment

In both the efficacy study and the pharmacokinetic study, cisplatin and MCA were injected as single bolus doses i.p. Cisplatin (Bristol-Myers Squibb, New York, NY) was given at a dose of 5 mg/kg and MCA (Lundbeck, Copenhagen, Denmark) at

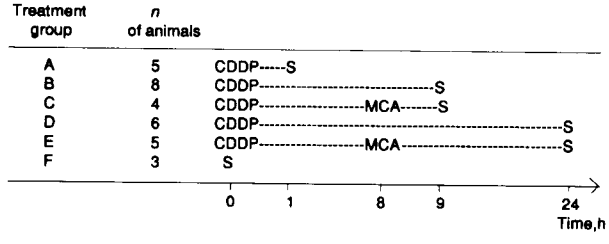

Figure 1. Experimental design of pharmacokinetic study, with treatment groups A–F, where F = untreated controls, CDDP = cisplatin treatment, 5 mg/kg i.p., MCA = MCA treatment, 2 mg/kg i.p., S = sampling of serum for platinum analysis and kidneys and tumors for analysis of cisplatin–DNA adducts.

a dose of 2 mg/kg, at 8 h after cisplatin. These doses and time interval have previously been shown to be optimal, with a substantial potentiation of the cisplatin cytotoxicity and minimal toxicity.[4]

Tumor volume measurement and growth curve analysis

Two orthogonal diameters were measured with vernier calipers on the tumors in the efficacy study. The tumor volume was calculated according to the formula[8]: volume = (length × width^2)/2. Tumors without growth from 3 days before treatment to the time of treatment or with starting volumes out of the range 50–500 mm^3 (diameters 4.6–9.8 mm) were excluded from growth curve analysis.

Relative tumor size (RTS, i.e. the tumor size at the time of measurement in relation to the tumor size at the time of treatment) was calculated.[9] To obtain a normal distribution of RTS values, the logarithmetized RTSs were used.

Specific growth delay (SGD) was calculated according to Berman and Steel.[10] From the curves of the log RTS versus time, the time interval for each tumor to double its volume (DT) was obtained. Tumor growth delay was calculated as the difference between the DT of the individual tumor and the mean DT of the control tumors. This difference was divided by mean DT of the controls, thus obtaining the SGD. SGD can be regarded as the number of DTs gained by the treatment. In order to avoid potential pitfalls,[11] the growth curves of each individual tumor were analyzed, rather than as mean values of groups.

Sample preparation

The mice included in the pharmacokinetic study were anesthetized with ether and exterminated by bleeding. The blood was allowed to clot and then centrifuged at 1500 r.p.m. for 10 min to obtain serum, which was stored at −70°C for later platinum analysis. One kidney and the largest tumor from each mouse were quickly removed and frozen at −70°C. Cryostat sections, 10 μm thick, were prepared on slides coated with poly-L-lysine and then stored at −70°C until staining for cisplatin–DNA adducts.

Platinum measurement

Serum-platinum was quantitated with flameless atomic absorption spectrophotometry. Sample pre-

paration was performed as previously described.[7] Measurements were made with a Varian Spectra AA-40Z with Zeeman background correction.

Cisplatin–DNA adduct analysis

An immunohistochemical staining technique for visualization of cisplatin–DNA adducts was performed as previously described,[12] using the NKI-A59 antiserum (gift from Dr den Engelse, Netherlands Cancer Institute, Amsterdam, The Netherlands) elicited against cisplatin–DNA interaction products.[13] A brown nuclear staining reaction was developed by peroxidase–anti-peroxidase complex and diaminobenzidine. Methyl-green was used as nuclear counterstain. Since only 19 slides could be stained in each batch and 30 animals were analyzed, the slides from tumor and kidney, respectively, had to be divided into two separate batches. The three control slides were included in both staining batches of each tissue type.

The staining reaction was quantitated with the computerized image analyzer CAS 200 (Cell Analysis System, Elmhurst, IL), using a two-color mask image technique with the quantitative nuclear antigen (QNA) software package. With a 40× objective lens, each microscopic field consisted of 50–100 cells. On each slide, 15–25 fields were measured. A total of 700–2000 cells per slide were thus analyzed. Results of the measurements are given as 'percentage positive nuclear area' (PNA). This procedure has been described and evaluated in a previous report.[12] To correct for non-specific staining that may vary between staining batches, the PNA values from the slides of the untreated control animals were subtracted from the remaining slides of that batch.

Statistics

In the efficacy study, differences between all four treatment groups were tested with one-way analysis of variance (ANOVA) and then the difference between the two treatment arms (cisplatin and cisplatin + MCA) was analyzed with Student's *t*-test. Since MCA has been shown to potentiate cisplatin in several previous studies,[3,4] a one-sided analysis was chosen.

Comparisons of plasma platinum concentrations and cisplatin–DNA adduct levels between the two treatment groups (cisplatin and cisplatin + MCA) were calculated by two-sided Student's *t*-test.

Results

Tumor growth was not affected by physiological saline nor by MCA alone (Figure 2 and Table 1). Cisplatin gave a significant but transient retardation of tumor growth. This retardation was further enhanced by MCA, in terms of difference in SGD between cisplatin alone and cisplatin + MCA ($p = 0.036$).

The pharmacokinetic profiles of total serum platinum (Figure 3) showed higher platinum concentrations in the animals treated with cisplatin + MCA compared with cisplatin alone. The difference was statistically significant at 9 h ($p = 0.03$) and almost significant at 24 h ($p = 0.057$).

The staining pattern of cisplatin–DNA adducts in kidneys and tumors was very similar to what has been previously described.[12] In kidneys the staining was strongest in the cortex, particularly in tubular cells in the outer cortex, whereas the medullary

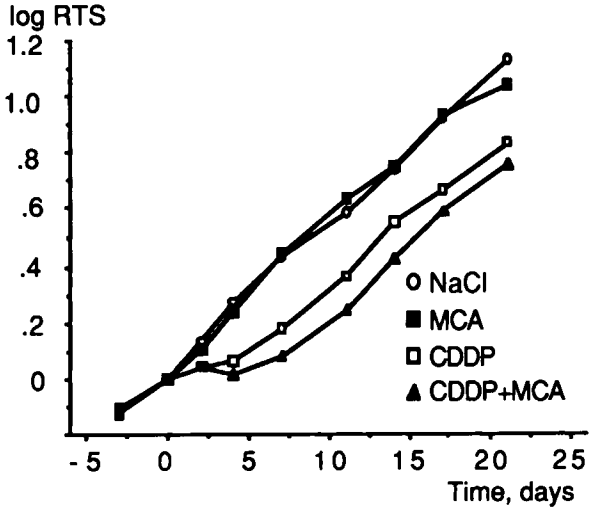

Figure 2. Mean log RTS, for tumors in nude mice treated with physiological saline i.p. (controls), 5 mg/kg of cisplatin i.p., 2 mg/kg of MCA i.p. or 5 mg/kg of cisplatin i.p. + 2 mg/kg of MCA i.p. given 8 h after cisplatin.

Table 1. SGD for tumors in nude mice treated with NaCl i.p., cisplatin 5 mg/kg i.p., MCA 2 mg/kg i.p. or cisplatin 5 mg/kg i.p. + MCA 2 mg/kg i.p. 8 h later. [the difference between cisplatin alone and cisplatin + MCA was $p = 0.036$ (one-sided *t*-test)]

Treatment	No. of tumors	SGD ± SE
NaCl	13	0.00 ± 0.12
Cisplatin	11	0.98 ± 0.22
MCA	12	0.01 ± 0.09
Cisplatin + MCA	16	1.49 ± 0.16
ANOVA		$p < 0.001$

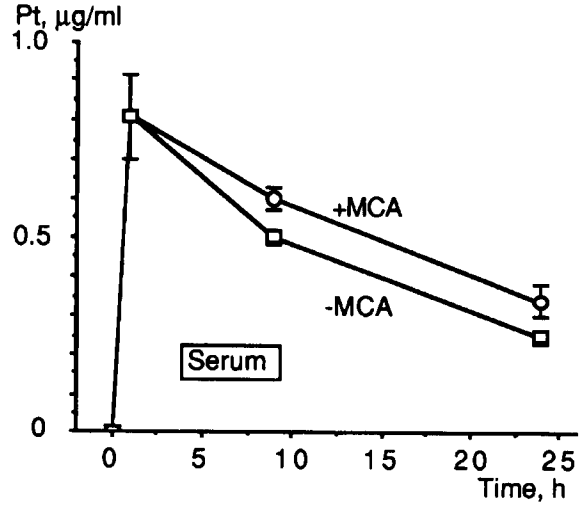

Figure 3. Serum-platinum levels in nude mice treated i.p. with 5 mg/kg of cisplatin, alone or in combination with 2 mg/kg of MCA, given 8 h after cisplatin. Data points are mean of four to eight animals ± SE.

staining was very low. In this study only the outer cortex was analyzed. The adduct staining of the tumors was generally weak. Positive nuclei were seen among all three cell components—morphologically intact tumor cells, necrotic tumor cells and stromal cells. Only morphologically intact tumor cells were analyzed.

The kinetic profiles of cisplatin–DNA adducts in kidneys showed an increase between 1 and 9 h, after which there was a steady-state in PNA values in the group treated with cisplatin alone (Figure 4). In the combined treatment arm there was an increasing staining intensity from 9 to 24 h, but the

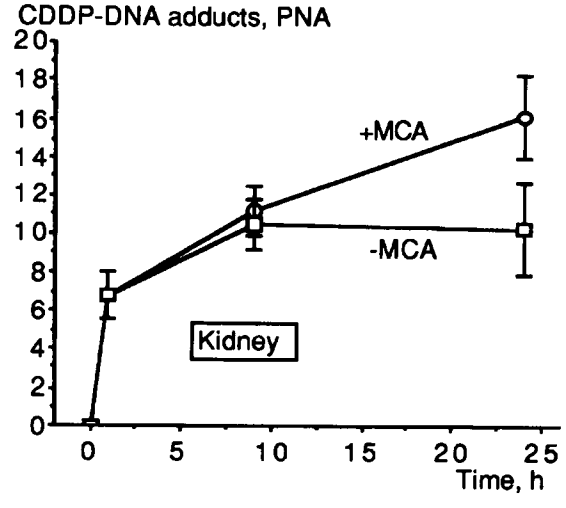

Figure 4. Cisplatin–DNA adduct levels in kidneys from nude mice treated i.p. with 5 mg/kg of cisplatin, alone or in combination with 2 mg/kg of MCA, given 8 h after cisplatin. Data points are mean of four to eight animals ± SE.

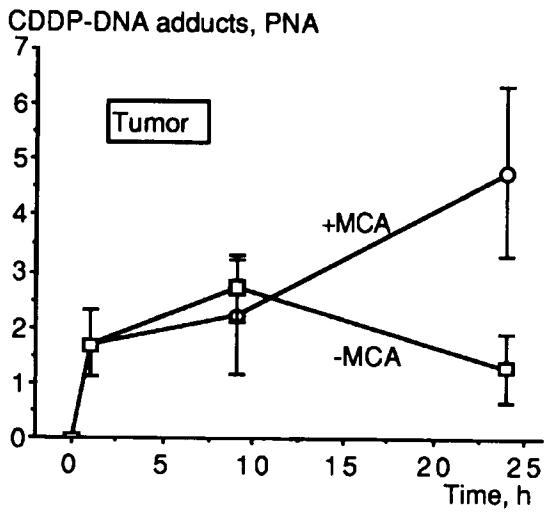

Figure 5. Cisplatin–DNA adduct levels in xenografted tumors from nude mice treated i.p. with 5 mg/kg of cisplatin, alone or in combination with 2 mg/kg of MCA, given 8 h after cisplatin. Data points are mean of four to eight animals ± SE.

difference between the treatment groups at 24 h was not statistically significant ($p = 0.1$).

In tumors there was also a gradual increase in adduct level from 1 to 9 h. Then, the PNA levels declined in the cisplatin alone group, but increased among animals receiving the combined treatment. The difference in PNA values at 24 h was statistically significant ($p = 0.04$) (Figure 5).

Discussion

This study confirms previous investigations[3,4] showing that the tumoricidal effect of cisplatin can be potentiated by the anti-emetic drug MCA. We have also shown that by combining cisplatin with MCA, the formation of cisplatin–DNA adducts is increased in tumor cells and to a lesser degree in kidney cells.

DNA is generally regarded as the main target for cisplatin as well as most other chemotherapeutic agents. Analysis of adducts and cross-links between cisplatin and DNA can be performed by different methods, e.g. atomic absorption,[14–15] ELISA[16,17] and immunohistochemistry.[12,18] In the present study, the cisplatin–DNA adducts were analyzed with quantitative immunohistochemistry, which allows topographic studies of adduct distribution on a cell-to-cell basis. The greatest disadvantage with this technique, as with all immunohistochemical assays, is that the stoichiometric relations are incompletely known. The results should therefore

be regarded as semi-quantitative rather than quantitative. However, we have previously found linear or almost linear relations between cisplatin dose and adduct staining levels expressed as PNA values.[7] This may indicate that a double PNA value represents approximately a 2-fold increase in cisplatin–DNA adducts.

Due to variations in staining levels between batches and differences in staining pattern in different tissues, the method should preferrably be used for comparisons between slides of the same tissue type, stained in the same batch.[12] Thus, in the present study conclusions could be drawn from the shape of the adduct profiles, whereas comparisons between PNA values of the two tissue types (kidney and tumor) should be avoided.

The drug doses used in the present investigation were based on previous studies with the same *in vivo* model and the same squamous cell carcinoma cell line (ÅB),[4] as in which a cisplatin dose of 5 mg/kg was the lowest due to give a substantial tumor growth delay in combination with MCA, with minor toxicity. Using this dose, the kidney slides displayed high intensity in the cisplatin–DNA adduct staining. In tumors the staining was fairly low and approaching the detection limit for the method,[7] which may have affected the statistical variation of our results.

MCA has previously[3,4] and in the present study been shown to potentiate the cytotoxic effect of cisplatin on xenografted squamous cell carcinomas *in vivo*. The present investigation also showed increasing levels of cisplatin–DNA adducts when combining cisplatin with MCA, which indicates enhanced formation of adducts, maybe accompanied by an inhibition of DNA repair. This assumption is in accordance with a study on the mechanisms of MCA's action both *in vitro* and *in vivo*, which showed MCA to inhibit repair of DNA damage but also to induce DNA strand breaks in mononuclear leukocytes.[5] A tentative explanation could be that DNA damage catalyzed by MCA makes the DNA more reactive with cisplatin, thus facilitating formation of additional cisplatin–DNA adducts.

Another factor that may have played a role was the change in cisplatin pharmacokinetics. We found slightly elevated concentrations of total platinum in serum among the animals receiving the combined treatment. This has not been reported before and the mechanism is unknown. It can be argued that the total platinum content in serum between 9 and 24 h after a cisplatin injection is of minor importance for adduct formation, since most of the drug will be protein-bound and inactivated by then. However, it cannot be excluded that also the free and active portion of serum-platinum was also increased, which in turn could lead to more of the active drug in the tissues and increased adduct formation.

We found increased levels of cisplatin–DNA adducts mainly in tumors, but the same tendency was found also in kidneys, suggesting that the effects on cisplatin-induced DNA damage is not tumor specific. In a recent human autopsy study,[19] Stewart *et al.* found higher platinum concentrations in kidney from patients having received MCA as an antiemetic. However, the addition of MCA to cisplatin has not been reported to enhance the toxicity of cisplatin. In studies on mice,[3] the mortality rate and body weight profiles were not affected by the addition of MCA, and no signs of increased nephrotoxicity has been reported. In patients treated with high-dose MCA against cisplatin-induced emesis, MCA did not alter the cisplatin-associated toxicity pattern.[20] Thus, there is no evidence that the observed tendency for increased cisplatin–DNA adduct levels in kidneys is enough to induce overt nephrotoxicity. It is still possible that the addition of MCA does increase the cisplatin-induced nephrotoxicity, but only to a minor extent and without any clinical consequences.

In summary, we have confirmed previous studies showing that MCA potentiates the tumoricidal effect of cisplatin. We have also observed that the levels of cisplatin in its probable target position, i.e. bound to DNA, seem to increase in tumors after addition of MCA. Thus, our findings may indicate that the sensitization of MCA on the cytotoxicity of cisplatin is mediated by increased formation of cisplatin–DNA adducts.

The fact that we found a tendency for adduct levels to increase more in tumors than in kidneys should be interpreted with caution, but it might indicate that MCA could increase the therapeutic index of cisplatin, thus making MCA a potentially useful sensitizer of cisplatin in the clinical setting. However, this investigation should mainly be regarded as a pilot study based on one dose level of drugs, a fixed time interval between drugs and with few sampling time points. To further elucidate the mechanisms of the sensitization of cisplatin action by MCA, cisplatin–DNA adducts and pharmacokinetics should be studied with higher cisplatin doses to improve the staining levels in tumors, at more sampling time points for a more complete description of the adduct kinetics and with complementary analysis of free serum-platinum to elucidate the pharmacokinetic alterations.

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