EDTA enhances the antitumor efficacy of intratumoral cisplatin in s.c. grafted rat colon tumors

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We have investigated whether EDTA, a calcium chelator, could improve the accumulation of platinum in tumors and enhance the antitumor efficacy by increasing drug diffusion through the extracellular tumor matrix. Intratumoral injection of 0.3 mg/kg cisplatin combined with 10 mg/ml EDTA in 2 ml saline serum led to tumor cure in four of eight rats and produced major tumor regression in the other animals. In contrast, intratumoral injection of cisplatin alone or EDTA alone had no antitumoral effect. EDTA increased platinum accumulation both *in vivo* and *ex vivo* in the PROb tumors. EDTA alone was cytotoxic at a concentration of 10 mg/ml, but neither increased platinum accumulation nor cisplatin toxicity on cultured PROb colonic cancer cells. We conclude that EDTA could be a useful and well-tolerated adjuvant for enhancing intratumoral cisplatin chemotherapy. *Anti-Cancer Drugs* **15:295–299 © 2004 Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2004, **15:**295–299

Keywords: cisplatin, EDTA, intratumoral chemotherapy

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Sponsorship: Supported by the 'Ligue Bourguignonne contre le Cancer'.

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Received 16 August 2003 **Revised form accepted** 26 November 2003

Introduction

Intratumoral chemotherapy (ITC) theoretically gives clinicians the opportunity to treat locally recurrent tumors when surgery, radiotherapy and systemic chemotherapy have failed. ITC increases both the residence time and the concentration of drugs in the vicinity of cancer cells [1]. Nevertheless, the antitumor efficacy of ITC remains insufficient, mainly due to the poor and heterogeneous distribution of drugs in solid tumors [2]. Tumor and host cells, vessels and molecules of the extracellular matrix are tightly associated in solid tumors [3]. Cancer cells are linked together by tight and adherent junctions, and are linked to matrix integrins, which contribute to the drug diffusion barrier. The plasma membrane of cancer cells constitutes the ultimate barrier before the drug reaches its cellular targets, usually DNA. Calcium is involved in the control of plasma membrane permeability and in the integrity of cell–cell or cell–matrix junctions, notably in epithelial tissues [4]. The calcium chelator EDTA is consequently used as a formulation additive which promotes the absorption of molecules through the epithelia. It increases the pulmonary absorption of fluorescein isothiocyanate dextrans, the s.c. diffusion of human epidermal growth factor, the corneal and conjunctival penetration of β-blockers, and the intestinal absorption of orally administered drugs through the modification of the attachment and permeability of the enterocytes [5–7]. The low molecular weight of EDTA (372 kDa) facilitates its diffusion through the extracellular matrix. EDTA induces intra- and extracellular calcium and magnesium depletion, as well as many physiological cell changes, such as the disruption of actin filaments and adherent junctions, and the activation of protein kinases [6,8].

In this study, we tested the hypothesis that EDTA could increase the intratumoral diffusion and, thus, the antitumor effect of intratumorally injected cisplatin on s.c. tumors in rats.

Material and methods

Animals

Female inbred BDIX rats, 4–6 months old, weighing 200–230 g, were bred in constant conditions of temperature, hygrometry and exposure to artificial light. Experimental protocols were consistent with the Guidelines on the Protection of Experimental Animals published by the Council of the European Community (1986) [9].

Cells and tumors

The DHD/K12 cells originated from a dimethylhydrazine-induced colon tumor in BDIX rats. The DHD/K12/PROb clone (subsequently known as PROb) was chosen for its consistent tumorigenicity when injected in syngeneic rats [10]. PROb cells were maintained in culture in Ham's F10 medium supplemented with 10% fetal bovine serum (FBS). Cells were detached with trypsin and EDTA, and centrifuged in the presence of

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complete culture medium with FBS to inhibit trypsin activity. A s.c. tumor was first obtained in the pre-thoracic area of a BDIX rat by injection of 1×10^6 PROb cells in 1 ml of Ham's F10 medium. Tumor pieces of approximately $2 \times 2 \times 2$ mm were cut from this initial tumor, then s.c. grafted in the pre-thoracic area of syngeneic BDIX rats.

Chemicals and drugs

Cisplatin (Cisplatyl; Aventis Laboratories, Paris, France) was obtained in its commercial form as a dry powder and was diluted in distilled water. Disodium ethylenediaminetetraacetate (EDTA; Titriplex; Sigma, St-Quentin-Fallavier, France) was diluted in a 9 g/l NaCl solution.

Assay of platinum content in cells and tumors

To measure the cellular accumulation of cisplatin, 1×10^5 cancer cells were seeded in 24-well tissue culture plates and cultivated for 72 h until confluent. Cells were incubated for 1 h at 37°C with 50 μg/ml cisplatin and various concentrations of EDTA diluted in 1 ml of a 9 g/l NaCl solution. Wells were rinsed twice with drug-free NaCl solution, and then the cells were trypsinized, centrifuged (1000 cycles/min) and lyophilized. The platinum cell content was measured by atomic absorption spectrometry (AAS) using a Zeeman atomic absorption spectrometer (Spectra-A, Fontenay aux Roses, France) [11].

To determine the platinum tumor concentration, tumor fragments were weighed and digested in a microwave digester (MLS-1200 Mega; Milestone, Sorisole, Italy). After dilution in distilled water, the platinum concentration of the digested samples was assayed as previously reported by AAS [12].

Treatment of animals

In order to evaluate the antitumor activity of intratumoral cisplatin and EDTA, treatment was performed 6 weeks after the s.c. graft when the tumor volume reached an average of $1373 \pm 50\ mm^3$. A single injection was made into the tumor with a 26-G needle. The injection volume was twice the volume of the tumor. Rats received either cisplatin, 10 mg/ml EDTA, a combination of both drugs or the NaCl solution as a control. The cisplatin dose was deliberately low (0.3 mg/kg of rat body weight, leading to a final concentration of 30–60 μg/ml, depending of the tumor volume) in order to avoid a systemic antitumor effect which is observed for a dose of cisplatin of 1 mg/kg or higher (unpublished data). The tumor volume (V) was calculated using the approximated formula $V = D^3/2$, with D as the mean of two orthogonal tumor diameters and was measured weekly with a caliper following treatment.

In order to measure the tumoral platinum content after an *in vivo* treatment, animals received an intratumoral injection of cisplatin (1 mg/kg of rat body weight, with the final concentration ranging from 100 to 200 μg/ml, depending of the tumor volume), alone or in combination with 1% EDTA. According to preliminary results, tumors were sampled 3 h after the injection, weighed and kept at –20°C until platinum assay by AAS.

For the *ex vivo* experiment, PROb tumors were excised, then incubated at 37°C for 3 h in a calcium-free Hank's balanced salt solution (HBSS) in the presence of 50 μg/ml cisplatin, alone or associated with 10 mg/ml EDTA, or with EDTA and 100 mg/ml $CaCl_2$. The tumors were weighed and kept at –20°C until platinum assay by AAS.

Drug cytotoxicity assay

In vitro cytotoxicity of cisplatin on PROb colon cancer cells was determined using a Crystal Violet colorimetric assay. Cells (1×10^5/well) were seeded and cultivated in 24-well tissue culture plates for 48 h until confluent. Cell incubation with cisplatin, alone or in association with EDTA, was performed for 1 h at 37°C in serum-free Ham's culture medium. The cells were trypsinized, washed twice and seeded (1×10^4/well) in 96-well tissue culture plates. After a 6 days of culture, the cells were fixed with pure ethanol, then stained with 1% Crystal Violet in distilled water. After flushing with excess dye, the Crystal Violet was eluted with 33% acetic acid. The optical density (OD) was read on an automatic photometer at a wavelength of 540 nm. The percent cell survival was determined as the OD in wells with treated cells/OD in the wells with untreated control cells × 100.

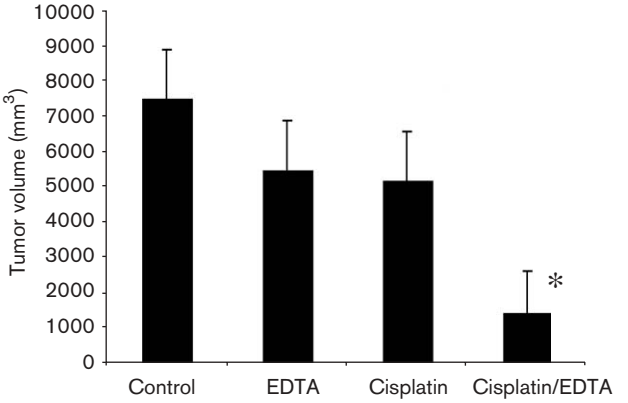
Statistical analysis

ANOVA was used with the Student–Neuman–Keuls test to localize the differences.

Results

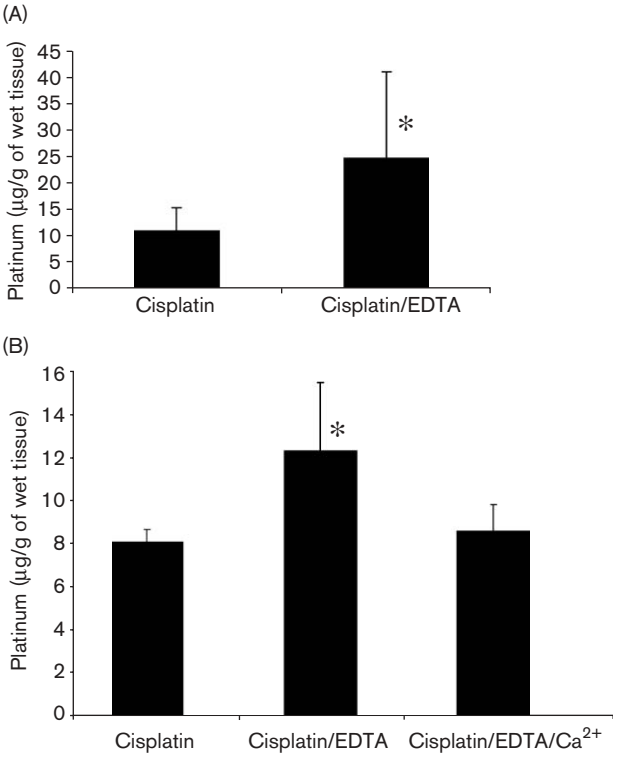
No animals were cured after intratumoral cisplatin or intratumoral EDTA alone. The mean final tumor volume did not differ in these groups from the control animals which received only the NaCl vehicle solution ($7500 \pm 2510\ mm^3$, Fig. 1). In contrast, intratumoral injection of cisplatin associated with 10 mg/ml EDTA had an obvious antitumor activity. Four of eight animals were definitively cured (no tumor recurrence when the animals were sacrificed 4 months later) and the four others had a significant reduction in the final tumor volume ($1430 \pm 964\ mm^3$, $p = 0.0085$). No treatment-related death occurred and general tolerance of the cisplatin–EDTA treatment was good. Limited local cutaneous necrosis occurred in cured animals with a complete healing obtained 6 weeks after the intratumoral injection. The mean platinum concentration after an *in vivo* intratumoral injection in the s.c. PROb tumors was higher when cisplatin was diluted in a 10 mg/ml EDTA solution (24.97 ± 16.59 μg/g) than when it was diluted in

Fig. 1



Antitumoral activity of intratumoral cisplatin and EDTA. Animals bearing 6-week-old s.c. PROb tumors (eight rats per group) received a single intratumoral injection of 0.3 mg/kg cisplatin diluted either in a NaCl solution (cisplatin) or combined with 10 mg/ml EDTA (cisplatin/EDTA). The other groups received intratumoral EDTA (EDTA) or the vehicle NaCl solution (control). The final tumor volume ± SD was measured 5 weeks after the treatment. *A significant difference with the control group ($p=0.0085$).

Fig. 2



Influence of EDTA on tumor platinum accumulation. (A) Animals (eight rats per group) were intratumorally injected with 1 mg/kg cisplatin alone (cisplatin) or combined with 10 mg/ml EDTA (cisplatin/EDTA). Tumors were excised 3 h later. *A significant difference between groups ($p=0.014$). (B) PROb tumors (five rats per group) were excised from animals then *ex vivo* incubated for 3 h at 37°C with 20 µg/ml cisplatin diluted in HBSS (cisplatin), HBSS supplemented with 10 mg/ml EDTA (cisplatin/EDTA) or 10 mg/ml EDTA and 100 µg/ml Ca^{2+} Cl_2 (cisplatin/EDTA/Ca). *A significant difference with the other groups ($p=0.02$).

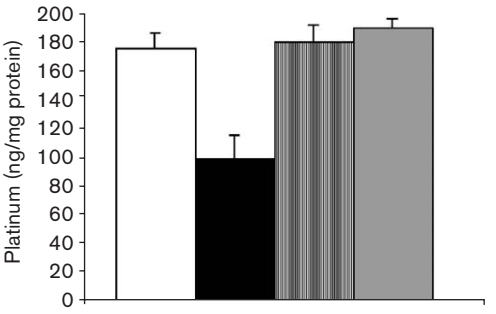
the NaCl vehicle (11.29 ± 4.53 µg/g, Fig. 2A). In order to investigate if the EDTA effect on platinum tumor accumulation was dependent on calcium chelation, an *ex vivo* incubation of PROb tumors was performed in calcium-free medium (HBSS), in the presence or absence of calcium (Fig. 2B). Calcium antagonized the potentiating effect of EDTA on platinum accumulation.

EDTA potentiation of platinum accumulation in PROb tumors was not related to any cellular effect since EDTA at all concentrations did not increase the *in vitro* platinum accumulation in cultured PROb cells (Fig. 3). Cell permeability to cisplatin was unchanged for concentrations of 0.1 and 1 mg/ml EDTA, and even lower for the 10 mg/ml concentration. The highest EDTA concentration of 10 mg/ml was toxic by itself on PROb cells in culture. Figure 4 shows only an additive cytotoxic effect of cisplatin and 10 mg/l EDTA, but not potentiation. The lower 0.1 and 1 mg/ml EDTA concentrations were nontoxic, but did not enhance the cisplatin toxicity.

Discussion

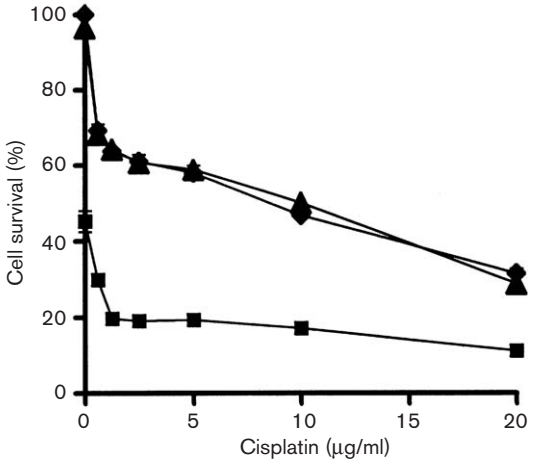
Our experimental results show that intratumoral EDTA enhances the tumor accumulation and the antitumor activity of cisplatin injected intratumorally in an animal model. In our experience, EDTA acts more at the tissue level than at the cellular level, since no potentiation of cisplatin accumulation or cytotoxicity by EDTA was observed in cultured cells. This is in contrast with the observation made by Park *et al.* [13], which showed that membrane permeability of cancer cells to rhodamine-123 and doxorubicin was lower in the presence of Ca^{2+} and

Fig. 3



In vitro accumulation of cisplatin in PROb colon cancer cells. Confluent cells were incubated for 1 h at 37°C with 50 mg/l cisplatin in the presence (dark bar: 10 mg/ml; vertical bar: 1 mg/ml; dashed bar: 0.1 mg/ml) or absence (open bar) of EDTA. Each value is the mean of four measurements ± SD.

Fig. 4



In vitro cytotoxicity of cisplatin and EDTA on PRObcolon cancer cells. Confluent cells were incubated for 1 h at 37°C with cisplatin alone (triangles) or associated with EDTA at 10 (squares) or 1 (diamonds) mg/ml. Each value is the mean of four measurements ± SD.

Mg^{2+}, and that doxorubicin uptake and cytotoxicity was enhanced in Ca^{2+}/Mg^{2+}-deprived medium. Part of the antitumor effect of the cisplatin–EDTA combination could be attributed to the cytotoxicity of 10 mg/ml EDTA alone, which added to the anticancer effect of cisplatin.

Increased platinum accumulation in the tumor mass when incubation was performed *in vivo* or *ex vivo* argues for an effect of EDTA on the tumor matrix. Drug diffusion through a tumor mass can be slow, depending on the histological type [3,14,15]. The extracellular matrix made of collagen, elastin fibers, proteoglycans and glycosaminoglycans forms a lattice network that hampers fluid mobility. Cohesive layers of cancer cells, notably in glandular tumors, build a mass which is barely penetrable due to the intercellular junctions. Intercellular junctions and cell adhesion to the extracellular matrix are strongly dependent on divalent ions, and could be released by EDTA. The epithelial permeability of polyethylene glycol 4000 on a Caco-2 cell monolayer was enhanced 14-fold, in comparison to the control, by 2.5 mg/ml EDTA. This effect was even higher in calcium-free conditions (up to 29 times the control value) [16]. The removal of extracellular calcium by EDTA stimulates protein kinase C (PKC), which dissociates adhesion molecules from cell–cell sealing and loosens junction strands. The use of a PKC inhibitor and extracellular calcium inhibited the EDTA effect and leads us to believe that EDTA opens the intercellular route by the stimulation of PKC through the removal of extracellular calcium [8,17]. In accordance with these findings, we noticed the disappearance of the potentiating effect on cisplatin tumor diffusion when calcium ions were added to a solution *ex vivo*. Calcium chelation by EDTA has been recently demonstrated to induce cadherin disassembly *in vivo* on pulmonary microvessels, as well as the loss of cell–cell interactions, which was proven by decreased VE-cadherin immunostaining [18]. However, we observed here neither obvious dissociation of cell–cell or cell–matrix junctions, nor modification of E-cadherin or α-catenin immunostaining after an *in vivo* injection of 10 mg/ml EDTA in PRObtumors (data not shown). The only consistent finding was extensive hemorrhaging in the tumor periphery that was probably related to the anticoagulant effect of EDTA. More precise methods than histological examination are required to confirm the hypothesis of an increased drug diffusion through the tumor by EDTA. Nevertheless, we can assume that the increase of the tissue homogeneity of platinum obtained thanks to EDTA allows better drug exposure of all tumor cells and a better antitumoral effect. In the group of eight animals treated with the EDTA–cisplatin solution, four were not cured. However, there was a significant reduction in the final volume of the four remaining tumors. Difficulties in obtaining perfect reproducibility during the injection and the variable architecture from one tumor to another can explain the persistent heterogeneity in the platinum distribution.

Another hypothesis should be evaluated to explain the cisplatin potentiation by EDTA. Because EDTA is a nucleophilic agent, it can be expected that chlorine atoms in the cisplatin molecule are exchanged with EDTA. The resulting molecule could have different pharmacological properties than the parent cisplatin, i.e. modified cell penetration, new intracellular ligands or enhanced formation of platinum–DNA adducts. In favor of such a hypothesis, Buraczewska *et al.* [19] have reported that ethylenediaminemalatoplatinum(II) complexes have greater antiproliferative properties than carboplatin. Moreover, lipid solubility of platinum group metals, an indicator of permeability through the plasma cell membrane, was shown to be enhanced by approximately 2-fold by EDTA [20]. The formation and pharmacological properties of cisplatin–EDTA complexes should be studied in more depth.

In conclusion, EDTA could be a useful and well-tolerated adjuvant for enhancing antitumoral activity of intratumoral cisplatin. These results open the way to other preclinical and clinical data. However, mechanisms of this potentiation remain unclear, and justify further investigations in other cellular and tumoral models.

Acknowledgments

We thank Jonathan Ewing for reviewing the manuscript.

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