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ORIGINAL ARTICLE

Detection of doxorubicin hydrochloride accumulation in the rat brain after morphine treatment by mass spectrometry

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Abstract
Purpose The blood–brain barrier discriminates the access of several molecules to the brain. This hampers the use of some drugs, as doxorubicin, potentially active for treatment of brain tumors. We explored the feasibility of active modification of the blood–brain barrier protection, by using morphine pretreatment, to allow doxorubicin accumulation in the brain in an animal model.
Methods Rats were pretreated with different doses of intraperitoneal morphine before injection of doxorubicin (12 mg/kg). Quantitative analysis of doxorubicin was performed by mass spectrometry. Acute heart and kidney damage was analyzed by measuring doxorubicin accumulation, LDH activity and malondialdehyde plasma levels.
Results The concentration of doxorubicin was significantly higher in all brain areas of rats pretreated with morphine than in control tissues ($P < 0.001$). This was evident only at therapeutic morphine dose (10 mg/kg, three times over 24 h), while lower doses (2.5 and 5 mg/kg) were not associated with doxorubicin accumulation. Pretreatment with morphine did not induce an elevation of LDH activity or of lipid peroxidation compared to controls.
Conclusion Our data suggest that morphine pretreatment is able to allow doxorubicin penetration inside the brain, by modulating the blood–brain barrier. This is not associated with acute cardiac or renal toxicity. These preliminary results will enable us to generate novel therapeutic approaches to refractory or recurrent brain tumors, and might be useful in other human diseases of the central nervous system in which molecules usually stopped by the blood–brain barrier may have a therapeutic impact.

Keywords Blood–brain barrier · Doxorubicin · Morphine · Rodent model · Mass spectrometry

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Introduction

Despite recent advances in the cure of childhood cancer, brain tumor still lags behind other cancer types [1, 2]. Although the reason for treatment failure may depend on several issues, inadequate efficacy of chemotherapy probably plays a major role, compared to what has been achieved in other cancer types.

Based on in vivo and in vitro studies, brain tumors might be expected to be sensitive to chemotherapy agents, as anthracyclines [3, 4]. Yet, most patients fail to achieve

adequate disease control due to insufficient cytoreduction of unresectable tumors, or develop tumor relapse despite apparent gross-total resection and/or irradiation [2].

One major obstacle to efficient tumor cytoreduction may be insufficient drug delivery to the tumor site. The blood–brain barrier (BBB) is a physical and metabolic barrier between systemic circulation and the brain. It maintains homeostasis and protects the brain from toxic insults. Yet, BBB may represent a real limiting factor to delivering chemotherapy agents within the brain tumor mass.

Morphine is the most used narcotic analgesic. In a recent report, Sharma and coworkers using a rodent model documented that morphine induced a transient alteration of BBB permeability to large molecules [5]. We hypothesized that a reversible modulation of the BBB by morphine might act as permeability enhancer for chemotherapy agents and designed an experimental rat model with the aim to achieve permeability of the BBB to doxorubicin by morphine pretreatment. Here, we demonstrate that doxorubicin concentration in the brain is greatly enhanced when doxorubicin is administered in the presence of therapeutic plasma levels of morphine.

Methods

Drugs and reagents

Doxorubicin (Adriblastina, Farmitalia Carlo Erba, Milan, Italy) 12 mg/kg in sterile H_2O was administered i.p. in a volume of 6 ml/kg [6]. Morphine (10 mg/kg) was dissolved in sterile saline and administered i.p. in a volume of 4 ml/kg.

Chemical standard of Doxorubicin hydrochloride (purity > 98%) was purchased from Sigma–Aldrich (Steinheim, Germany); the internal standard, Doxorubicin-$^{13}C_4$ (99.8% D purity) was from Medical Isotopes Inc. (Pelham, USA). Stock solutions of both were made in HPLC grade methanol (2,000 mg/l). Further dilutions were made using HPLC grade water. All chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

Animals

Male adult Wistar rats (260–280 g) were used (Harlan Italia, Milano, Italy). All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC). All efforts were made to minimize animal sufferings and to use only the number of animals necessary to produce reliable scientific data. Rats were randomly allocated to one of the following experimental groups (5 animals per group):

Group 1 (D + M): on day 1, rats were treated twice with morphine (10 mg/kg, i.p., at 10.00 a.m. and again at 5.00 p.m.); on day 2 at 10.00 a.m., they received a third dose of morphine; 1 h later they received i.p. doxorubicin.
Group 2 (D): rats were treated with doxorubicin alone.
Group 3 (Ctr): naïve rats received no drugs.

Neurological test

Neurological evaluation of motor sensory functions was carried out 10 days after doxorubicin administration on two groups of rats: (a) rats treated as group 1 animals ($n = 4$) and saline-treated rats ($n = 4$) as controls. The examiners were blind as to the procedure that the rat had undergone. Evaluations were performed in the afternoon. The neurological examination consisted of six tests: (1) spontaneous activity; (2) symmetry in the movement of four limbs; (3) forepaw outstretching; (4) climbing; (5) body proprioception; (6) response to vibrissae touch. The score assigned to each rat at completion of the evaluation equals the sum of all six test scores. The final minimum score was 3 and the maximum was 18 [7, 8]. Rats were weighted every day.

Collection of tissues

One hour after administration of doxorubicin, rats were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.). The heart was exposed and blood was withdrawn by intracardiac puncture, collected in a preheparinized plastic tube, plasma was separated from blood cells. Animals were then perfused transcardially with ice-cold saline (500 ml, 50 ml/min). At the end of perfusion, the cerebral hemispheres, the cerebellum, and brainstem were dissected; heart and kidney were collected; all tissues were rinsed in ice-cold saline and transferred to screw-capped plastic tubes. All tissues were immediately frozen on dry ice and kept at −80°C until assay.

Quantitative analysis of doxorubicin by mass spectrometry

Doxorubicin determination on plasma

To quantify plasma concentration of doxorubicin, samples (25 µL) were spotted on filter paper (903®, Whatman GmbH, Dassel Germany) and air-dried. Dried plasma spots were punched and two 3.2-mm circles (containing about 1.75 µL of plasma each) were extracted together with 200 µL of 20/80 water/methanol solution containing 10 µmol/l of $^{13}C_4$-doxorubicin. Samples were put on an orbital shaker and kept at 37°C for 20 min.

For the setting up of this study, a pooled mixture of plasma samples from untreated rats was spiked with increasing concentrations of doxorubicin and 25 µL was spotted on 903® filter paper.

Tissue sample preparation

Thawed brain sections (cerebral hemispheres, brainstem, and whole brain) were weighed and spiked with 2 µL of $^{13}C_4$-doxorubicin (10 µmol/l) directly injected into the tissue. After 10 min, the sections were diluted with 2.5 ml H_2O. Sample homogenization was performed using a mechanical homogenizer (UltraTurrex, IKA-Werke, Staufen, Germany) followed by 10 min of intermittent ultrasonication in the ice bath. Samples were defatted using 2 ml of chloroform with vigorous shaking for 5 min, followed by centrifugation at 3,000 rpm for 10 min. The aqueous upper phase was transferred to a new tube, and the chloroform extraction step was repeated 6 times. After the last extraction acetonitrile (400 µL) was added and precipitates removed by centrifugation at 13,200 rpm for 3 min, the samples were dried out and rehydrated with acidified methanol (0.1% formic acid, v/v) 300 µL. To determine the extraction yield of doxorubicin, brain tissue samples from an untreated rat were spiked with doxorubicin (5 µmol/l) before and after extraction process.

Mass spectrometry

An Applied Biosystems-Sciex (Toronto, Canada) API 4000 bench-top TurboIonSpray-Triple-Quad Mass Spectrometer (MS/MS) was used for this study. The ion source operated under positive ion mode (5,500 V).

Declustering potential (DP), collision exit potential (CXP) and collision energy (CE) were automatically optimized for doxorubicin and $^{13}C_4$-doxorubicin. The resulting DP was 35 V. Optimal CE and CXP were found at 16 and 10 V, respectively (Fig. 1).

Quantitative analysis was undertaken using an HPLC Series 1100 Agilent Technologies (Waldbronn, Germany). Liquid chromatography was performed using a Phenomenex Synergi 4 µm Polar-RP 80A 4 µm, 2 × 150 mm HPLC column (Phenomenex, Anzola Emilia, Italy) injecting 5 µL of the extracted sample. Column flow was 0.2 ml/min using an isocratic aqueous solution of 80% methanol containing 0.1% formic acid. The eluent from the column was directed to the TurboIonSpray probe without split ratio.

LDH activity and tissue lipid peroxidation

Lactate dehydrogenase (LDH) activity was measured in rat plasma using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) and absorbance read at 490 nm.

Tissue lipid peroxidation in terms of malondialdehyde (MDA) production (thiobarbituric acid-reactive species) was determined as previously described [9].

Statistical analysis

Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test as a post hoc analysis or by Student's *t* test, as appropriate. A *P* value of 0.05 or less was taken as the criterion for statistically significant difference.

Results

The concentration of doxorubicin within the brain tissue was significantly higher in rats pretreated with morphine than in those treated with doxorubicin alone. Doxorubicin was undetectable in control samples from untreated rats (Fig. 2).

Doxorubicin concentration was significantly higher in all the brain areas under study of animals pretreated with morphine (Fig. 3).

Cerebral hemispheres: the mean concentration of doxorubicin in the cerebral hemispheres of rats receiving doxorubicin alone was 5.88 ± 0.34 ng/g fresh tissue ($n = 5$) versus 18.8 ± 1.01 ng/g fresh tissue in those treated with doxorubicin plus morphine ($n = 5$), while control animals had no doxorubicin (0.002 ± 0.001 ng/g fresh tissue, $n = 3$). The estimated limit of detection of doxorubicin (signal-to-noise ratio > 3) in the brain was 1 ng/g fresh tissue, and the limit of quantitation (signal-to-noise ratio > 5) was 2 ng/g fresh tissue. This difference was statistically significant (one-way ANOVA F[2, 10] = 168.3, $P < 0.0001$; *** $P < 0.001$ versus all other groups, Newman–Keuls post hoc test).

Cerebellum: the mean concentration of doxorubicin in the cerebellum of rats receiving doxorubicin alone was 5.18 ± 0.38 ng/g fresh tissue ($n = 5$) versus 15.94 ± 0.371 ng/g fresh tissue in those treated with doxorubicin plus morphine ($n = 5$), while control animals had no doxorubicin ($n = 3$). This difference was statistically significant (one-way ANOVA F[2, 10] = 482.0, $P < 0.0001$; *** $P < 0.001$ versus all other groups, Newman–Keuls post hoc test).

Brainstem: the mean concentration of doxorubicin in the brainstem of rats treated with doxorubicin alone was 5.71 ± 0.38 ng/g fresh tissue ($n = 5$) versus 14.6 ± 0.25 ng/g fresh tissue in those treated with doxorubicin plus morphine ($n = 5$). Control animals had no detectable doxorubicin (0.001 ± 0.001 ng/g fresh tissue, $n = 5$). This difference was statistically significant (one-way ANOVA F[2, 10] = 521.2, $P < 0.0001$; *** $P < 0.001$ versus all other groups, Newman–Keuls post hoc test).

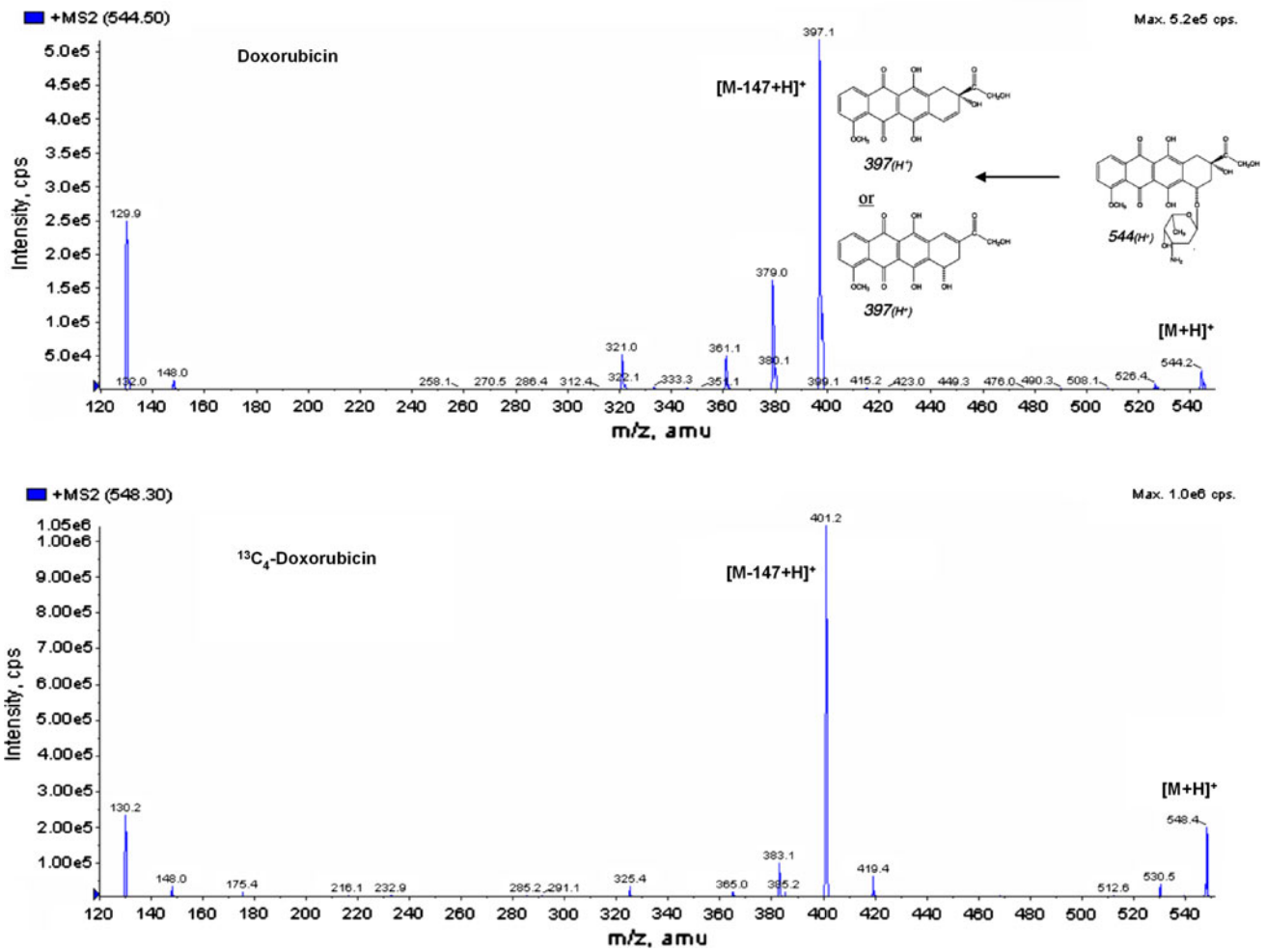

Fig. 1 MS/MS spectrum obtained by fragmenting the precursor ion (544.4 Th) of doxorubicin and the precursor ion (548.4 Th) of internal standard ($^{13}C_4$-doxorubicin) under the above described conditions. From these experiments, the resulting ion-pair transition for the quantitative experiment (MRM) is 544.4 > 397.2 for doxorubicin and 548.4 > 401.2 for $^{13}C_4$-doxorubicin. The peaks corresponding to doxorubicin and internal standard eluted from the chromatographic column at 4 min. No interferences were revealed at the same retention time. The choice of a binary mobile phase was made considering the chromatographic column conditions

Pretreatment of rats with lower doses of morphine (5 or 2.5 mg/kg, three times in 24 h, i.p.) failed to facilitate doxorubicin entry into the brain ($n = 3$; data not shown).

Mean plasma levels of doxorubicin in animals pretreated with morphine (12.89 ± 0.71 μmol/l plasma, $n = 5$) did not differ from that found in rats receiving doxorubicin alone (13.26 ± 2.12 μmol/l plasma, $n = 5$) ($P = 0.8711$, Student's t test) (Fig. 4a).

Cardiac and renal toxicity after treatment with an anthracycline is a matter of great concern. We therefore investigated whether pretreatment with morphine might increase the doxorubicin concentrations in the heart and kidney, thus predicting increased toxicity in these two target organs. Pretreatment with morphine did not increase the levels of doxorubicin in either tissue at 1 h after administration ($P > 0.05$, Student's t test, Fig. 4b, c).

We addressed the issue of cardiac and renal toxicity by analysis of plasma LDH activity and MDA levels. Doxorubicin induced a 60% increase in plasma LDH activity 1 h after treatment in treated versus control rats (Table 1, P = n.s.). No difference in LDH activity was found between rats treated with doxorubicin alone and those receiving morphine plus doxorubicin.

Mean MDA plasma levels were not statistically different among the three groups (Table 1).

In order to clarify whether the concomitant administration of doxorubicin and morphine might cause any late neurological toxicities/mortalities, we followed a group of rats for 10 days after the administration of the two drugs. We did not observe any clear sign of clinical toxicity nor any of the animals died after treatment. Rats treated with doxorubicin plus morphine did not show any neurological deficit

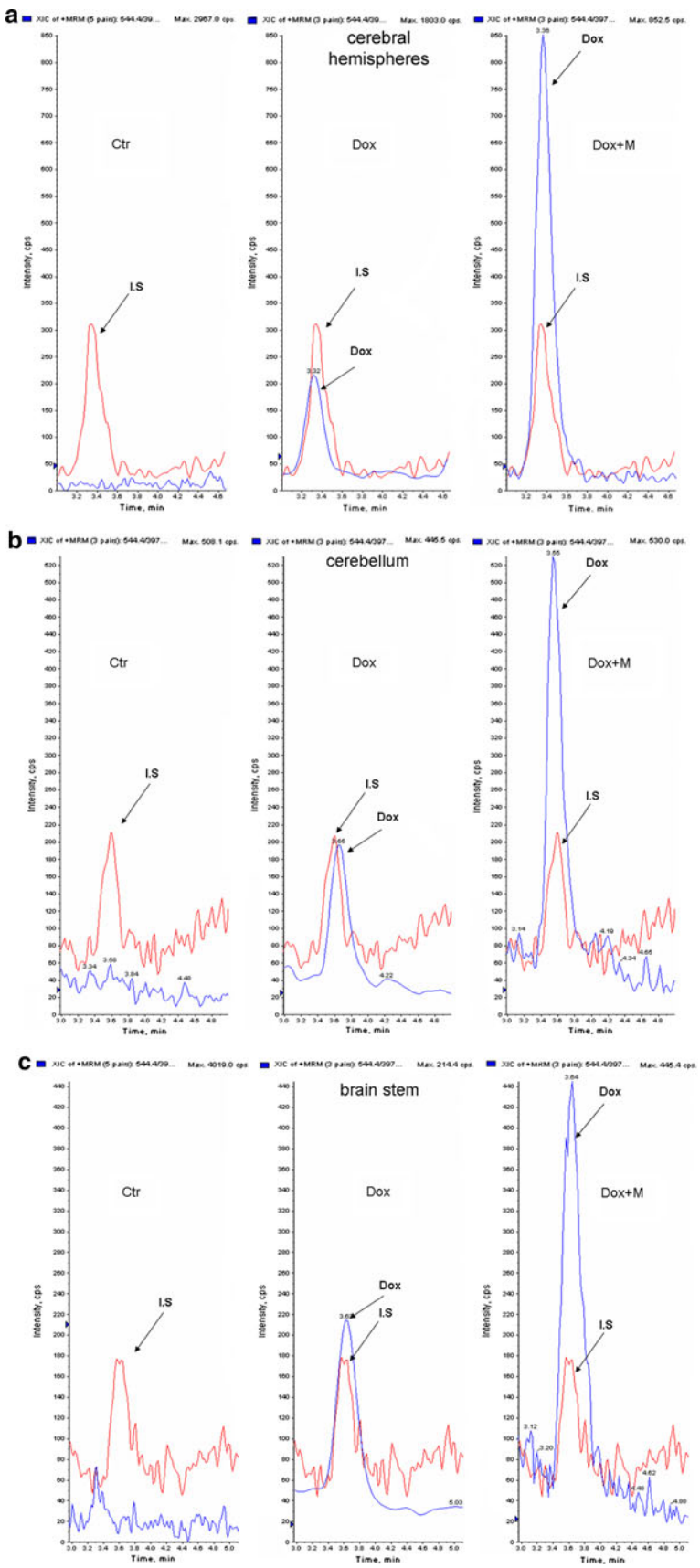

Fig. 2 Extracted ion chromatograms of doxorubicin and internal standard (IS) in cerebral hemisphere (**a**), cerebellum (**b**), and brainstem (**c**).
A doxorubicin signal was very close to baseline in all control samples and therefore not detectable

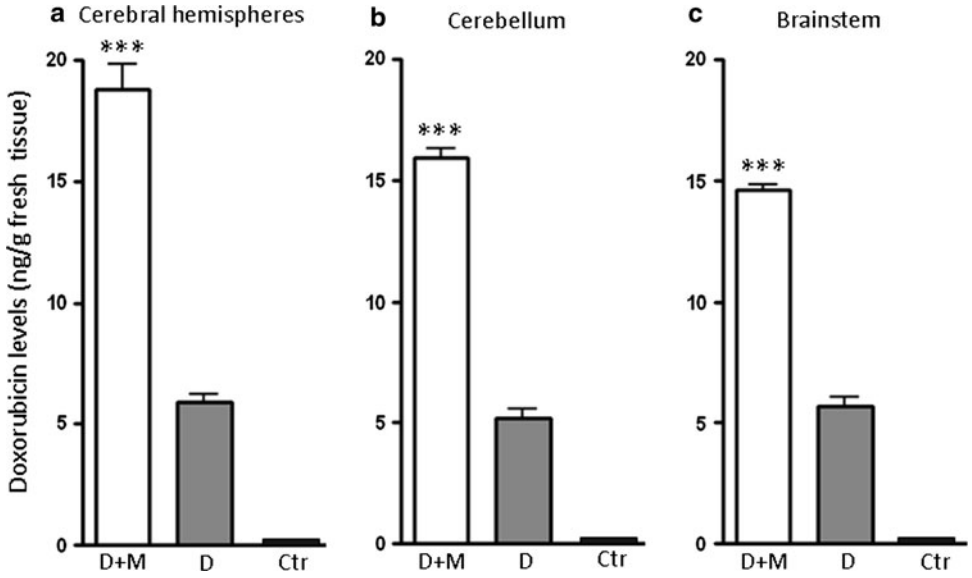

Fig. 3 Levels of doxorubicin in cerebral hemispheres (**a**), cerebellum (**b**), and brainstem (**c**) of the rat 1 h after a single administration of doxorubicin (12 mg/kg, i.p.). Doxorubicin was administered to different groups of rats either alone (D, *grey bars*, $n = 5$) or after pretreatment with 3 doses of morphine (10 mg/kg each, three times in 24 h, D + M, *open bars*, $n = 5$). Control animals (Ctr) received neither drug (*black bars*, $n = 3$). Tissue levels of doxorubicin are expressed as μg/g fresh tissue (mean ± SEM). Statistical analysis was performed separately within each brain area investigated. Significance: *** $P < 0.001$ versus all other groups (one-way ANOVA and Newman–Keuls post hoc test). Levels of doxorubicin in the cerebral hemispheres of rats treated with morphine plus doxorubicin were significantly higher than in cerebellum or brainstem ($P < 0.01$, one-way ANOVA and Newman–Keuls post hoc test)

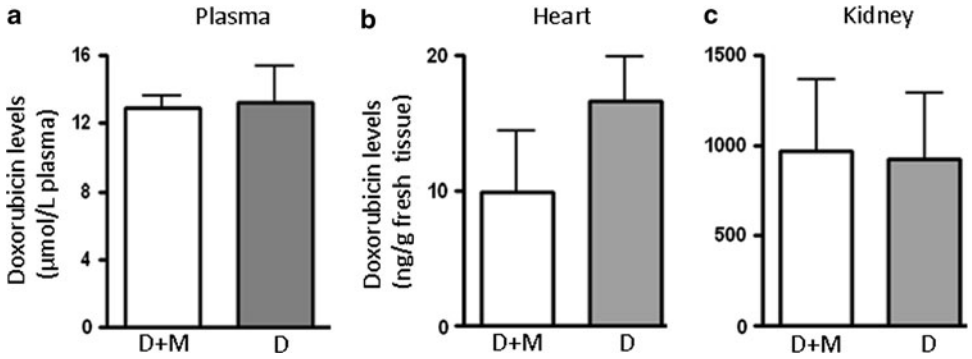

Fig. 4 Levels of doxorubicin in plasma (**a**), heart (**b**), and kidney (**c**) of the rat 1 h after a single administration of doxorubicin (12 mg/kg, i.p.). Doxorubicin was administered to different groups of rats either alone (D, *grey bars*) or after pretreatment with 3 doses of morphine (10 mg/kg each, three times in 24 h, D + M, *open bars*). *Panel A* plasma levels of doxorubicin are expressed as μmol/l plasma, D + M: $n = 5$; D: $N = 5$. *Panels B* and *C* Tissue levels of doxorubicin are expressed as μg/g fresh tissue. *Panel B* D + M: $n = 3$, D: $n = 3$. *Panel C* D + M: $n = 3$, D: $n = 3$. Data presented are the mean ± SEM. Statistical analysis was performed separately on data from each tissue investigated. Statistical analysis showed no significant differences between each pair of data ($P > 0.05$, Student's t test)

in comparison with the control group 10 days after treatment (final maximum score ± SEM: 17.50 ± 0.29 vs. 17.75 ± 0.25). Rats treated with morphine plus doxorubicin had significant body weight loss for the first 6 days after treatment, after which they started to gain weight.

Discussion

The main result of our study is that pretreatment with morphine at therapeutic dose allows delivery of doxorubicin within the brain tissue, which is otherwise precluded by the blood–brain barrier. Penetration of doxorubicin appears to be unrestricted to the different areas of the brain.

A prerequisite for the efficacy of a cytotoxic drug for the treatment of cancer is its capacity to reach the tumor cell at therapeutic concentrations. For brain tumors, this goal is usually hampered by BBB, a structure with the delicate duty to preserve, as far as possible, the integrity of brain tissue from chemical or infectious hazards.

Recently, Sharma et al. reported that rats treated with morphine exhibited leakage of albumin in several brain

Table 1 Levels of LDH activity and MDA production in plasma of control rats, rats treated with doxorubicin alone or after pretreatment with morphine

Group	LDH (U/ml plasma)	MDA (nmol/ml plasma)
Control	0.72 ± 0.08	323.2 ± 64.7
Doxorubicin	1.15 ± 0.26	294.0 ± 60.1
Doxorubicin + morphine	1.16 ± 0.15	290.0 ± 73.2

Doxorubicin was administered to different groups of rats either alone (12 mg/kg, i.p., doxorubicin, $n = 7$) or after pretreatment with 3 doses of morphine (10 mg/kg each, i.p., three times in 24 h, Doxorubicin + Morphine, $n = 6$). Control animals ($n = 6$) received neither drug

Values are the mean ± SEM. Statistical significance between means was analyzed using one-way ANOVA followed by the Newman–Keuls post hoc multiple comparison test. Differences among groups were not statistically significant

areas. This phenomenon was much more pronounced on the second and third day after morphine withdrawal, and was associated with ultrastructural changes in the nerve and glial cells [5]. The authors hypothesized that neurochemical mediators and vasoactive agents act on the vessels through signal transduction pathways leading to the opening of the BBB. Based on these data, we decided to investigate the possibility of morphine-mediated active BBB permeabilization.

Morphine is a substrate of the MDR1 isoform of P-glycoprotein (P-gp) efflux transporter, which very efficiently removes several molecules and drugs from the CNS, thus limiting their entry into the brain. Morphine-mediated "accumulation" of doxorubicin in the brain might result from its reduced efflux mediated by P-gp at the level of the BBB cells [10]. P-gp is localized mostly at the apical side of brain endothelial cell membrane. P-gp substrates flowing into the endothelial cells are rapidly pumped back into the blood, thus preventing them from entering the brain. This hypothesis is supported by evidence that P-gp blockade by specific inhibitors results in a significantly increased brain concentration of some antiepileptic drugs [10]. Whether alternative or additional mechanisms may mediate the impact of morphine and other opioid drugs on the BBB remains to be elucidated further [11–13].

Data on the effects of chronic exposure to morphine are conflicting [14, 15]. In rats chronically treated with morphine, in situ perfusion of sucrose did not allow its penetration into the BBB [15]. These data might be considered at odds with our results. Yet, we documented that an antiblastic drug—doxorubicin—accumulated in the brain and was detected by mass spectrometry, a highly precise, reliable, and sensitive method. Furthermore, several studies demonstrate that morphine and doxorubicin use the same efflux channels [10, 16], whereas sucrose crosses BBB by diffusion. We consider these data not to be an argument against our results.

On the other side, our group has recently reported unexpected respiratory distress as enhanced toxicity of dimethylsulfoxide (DMSO) on the central nervous system of children receiving morphine during hematopoietic stem cell transplantation. While this finding was apparently unexplained, we now consider that DMSO may have had undesired access to the brain tissue due to concurrent morphine-mediated temporary alteration of the BBB [17].

Delivering specific drugs beyond the BBB may open novel therapeutic approaches for several human diseases [18]. Pioneer experience in the treatment of childhood leukemia brought to intrathecal injection [19]. Yet, intrathecal chemotherapy can be adopted with great caution and only for a very limited number of drugs. Inadvertent intrathecal administration of anthracycline turned into catastrophic toxicity [20, 21]. Intracarotid infusion of hyperosmolar mannitol has been used to disrupt the BBB with the aim to increase the delivery of water-soluble agents into the brain and peritumoral areas [22]. Although this method gave some promising results, it requires an invasive surgical approach, with high risk of adverse effects that limit its potential clinical application on a large scale. More recently, nanoparticles have been adopted to enhance the transport of therapeutic agents across the BBB, providing refined drug delivery [23].

Doxorubicin accumulation was achieved after pretreatment with therapeutic dose of morphine. When we tried to document this phenomenon with lower doses of morphine pretreatment (25 and 50% dose), we did not document any accumulation compared to control rats, not pretreated with morphine. Thus, permissive effect of morphine appears to be active only beyond a threshold dose. Moreover, our data demonstrate that the concentration of doxorubicin after morphine pretreatment was significantly higher in cerebral hemispheres than in cerebellum and brainstem (Fig. 3). This result may depend upon a different structure of BBB or on a different distribution of P-gp isoforms in these brain areas, making the brainstem less accessible to many molecules.

Although doxorubicin is a very effective anti-cancer drug, its cardiac toxicity has to be considered as a potential limitation to unrestricted use. To evaluate whether doxorubicin toxicity might be enhanced by co-administration of morphine, thus leading to dangerous acute and/or chronic side effects, we quantified doxorubicin levels in heart and kidney of morphine pretreated rats. Co-administration of morphine did not increase the levels of doxorubicin in either tissue 1 h after administration. We also evaluated plasma LDH activity and MDA levels, as markers of acute cardiotoxicity, and found no difference between rats treated with doxorubicin alone and those also receiving morphine (Fig. 4).

In conclusion, our preliminary results indicate that morphine pretreatment mediates doxorubicin delivery beyond the BBB to the brain, in the absence of signs of increased acute toxicity. This finding might provide the rationale for clinical applications in the treatment of refractory brain tumors and pave the way to novel applications of active but currently inapplicable drugs. It remains to be assessed whether this observation will also facilitate studies exploring the brain delivery of other active agents, such as monoclonal antibodies or other macromolecules for the treatment of brain tumors or other human brain disease.

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