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International Journal of Pharmaceutics 270 (2004) 75–82
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Comparative study between the interaction of dephosphorylated amifostine (WR-1065) and amoxicilline with pBR322 in absence and presence of cisplatin by AFM

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Received 26 April 2003; accepted 10 October 2003

Abstract

The effect of the amoxicilline as a possible cisplatin-action protector on pBR322 DNA has been visually studied by atomic force microscopy in comparison with the modifications caused by the controversed cisplatin protector amifostine. Incubation of amoxicilline with the plasmid DNA showed aggregation and compaction of DNA. Cisplatin incubated in the same conditions with DNA produced kinks and super-coiling of the circular form. In the case of previous treatment of DNA with amoxicilline, the characteristic effect of cisplatin is only partially observed. The amoxicilline seemed to control the action of cisplatin on DNA. The initial effect of dephosphorylated amifostine (WR-1065) when this protector was incubated with the plasmid was also the formation of aggregates and the compaction of DNA. However, addition of cisplatin successively after 1 and 2 h showed the characteristic modification caused by cisplatin but only in a decreasing percentage of molecules of DNA. Both molecules, amoxicilline and WR-1065 seem to control the strong direct action of cisplatin on DNA. This effect can justify the role as protecting agent of amifostine on side effects caused by cisplatin and can open new possibilities to other agents like amoxicilline.

Keywords: Amifostine; Amoxicilline; Cisplatin toxicity; Cisplatin chemoprotection; Cisplatin AFM study

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II)) is a well-known anti-tumor compound. It has been demonstrated that in proliferating cells the intracellular target of the metal complex is DNA (Rosenberg et al., 1969; Lippert, 1999; Reedijk, 1999; Jamieson and Lippard, 1999; Giandomenico et al., 1999). In vitro and clinical studies suggest that the presence of sulphur-containing compounds in cisplatin solutions affects not only the physicochemical properties but the modulation of cisplatin-induced toxicity. The hydrosulphur groups are avid nucleophile for platinum but their easy reversible oxidation-reduction can alter the intracellular metabolism. In a previous work (Pérez-Benito et al., 1995) we have studied kinetically the interaction between cisplatin and biological thiols, among them, penicillamine. Complexes of penicilliamine and Pt(II) have also been studied (Cervantes et al., 1998) and it has been demonstrated that the coordination of Pt(II) involves the amide and thioeter groups forming a five-membered ring.

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doi:10.1016/j.ijpharm.2003.10.035

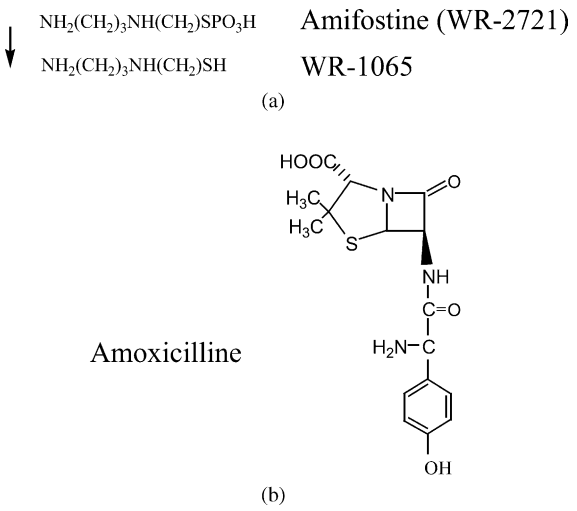

Fig. 1. (a) Pathway of dephosphorylation of amiforstine, Ethiol (WR-2721). (b) Amoxicilline.

On the other hand, in the literature the formation of complexes between Pt(II) and β-lactam antibiotics as ampicilline also involving N and S atoms is described (Grochowski and Samochocka, 1991; Samochocka et al., 1997). It would be very interesting then, to find sulphur-containing compounds that when interacting with cisplatin protect it from its toxicity without affecting its anti-tumour efficiency. In this attempt we have started a study of the chemical interaction of amoxicilline, a sulphur-containing compound, with cisplatin.

Amifostine [*S*-2-(3-aminopropylamino)ethyl-phosphorothioic acid, WR 2721, Ethyol®] (Fig. 1) is one of the chemoprotective agents in the modulation of cisplatin induced toxicity. In pre-clinical studies amifostine appeared to reduce the incidence of cisplatin-induced nephrotoxicity without reducing its anti-tumour activity (Yuhas and Culo, 1980; Yuhas et al., 1980; Jordan et al., 1982; Treskes et al., 1992; Johnsson and Wennerberg, 1999; Bergstrom et al., 1999; Castiglione et al., 1999). In clinical trials, amifostine appeared to reduce the incidence of cisplatin-induced nephrotoxicity and hematotoxicity compared to historical data (Glover et al., 1986, 1987, 1989; Hartmann et al., 2000a,b). The influence on cisplatin-induced ototoxicity is still unclear although the use of sulfur or sulfydryl-containing compounds as protective agents in co-treatment with cisplatin has been recently described (Smoorenburg et al., 1999; Moldoon et al., 2000). The influence of amifostine on the pharmacokinetics of cisplatin in cancer patients has been studied (Korst et al., 1998; Hospers et al., 1999). However, the addition of amifostine in the treatment schedule only produces minor changes in the pharmacokinetics of cisplatin and has no significant impact on the efficacy of cisplatin as already confirmed by clinical studies. Many attempts have been made at understanding the mechanism by which WR-2721 reduces nephrotoxicity. It is generally accepted that WR-2721 acts as a pro-drug which is converted into the active species WR-1065 when dephosphorylated by the membrane protein alkaline phosphatase (Fig. 1). The uncharged free thiol species WR-1065 is considered to be the true species taken up by the cell and passes the membrane by passive diffusion (Calabro-Jones et al., 1988; Souid et al., 1999; Bonner and Shaw, 2000). The selective uptake of WR-1065 in normal cells might be related to the lower levels of alkaline phosphatase of tumor cells (Shaw et al., 1988; Santini and Giles, 1999; Renner et al., 1999). Furthermore, the pH of normal cells is neutral while the pH of tumors is slightly acidic and this fact can help the selective behavior (Van der Vijgh and Peters, 1994). Experiments using model systems indicate that WR-1065 substitutes the Pt-methionine bond very slowly, but is a very potent inhibitor of DNA platination (Treskes et al., 1992). This suggests the direct interaction of WR-1065 with cisplatin preventing its toxicity in normal cells. Theoretical studies have simulated the radioprotection of the aminothiol WR-1065, active metabolite of amifostine(WR-2721), and the hydrogen abstraction by C4′ of DNA deoxyribose radical has been modeled (Broch et al., 2001).

Thus, the aim of this study was to compare the effect of amoxicilline and desphosphorylated amifostine WR-1065 on the interaction of cisplatin with DNA by using Tapping Mode Atomic Force Microscopy (TMAFM). In previous studies (Cervantes et al., 1997, 1999; Onoa et al., 1998; Onoa and Moreno, 2002) we have imaged the modifications introduced in DNA by Pt and Pd complexes. The incubation of amoxicilline or WR-1065 with DNA and further addition of cisplatin at different times has allowed us to image the behaviour of the two compounds as possible DNA protecting agents.

2. Materials and methods

2.1. Materials

Cisplatin was purchased from Johnson Matthey (Reading, UK). Amifostine and pBR322 were purchased from Boehringer (Mannheim, Germany) and amoxicilline from Centro Genesis (Barcelona, Spain). HEPES was acquired from ICN (Barcelona, Spain) and used without further purification. The solvents were purchased from Fluka (Madrid, Spain). $MgCl_2$ and KCl came from Merck (Darmstad, Germany).

2.2. Preparation of adducts of DNA

The commercial plasmid pBR322 was previously heated in a bath at 60 °C to ensure the presence of a major percentage of the circular relaxed form as it could be confirmed by the electrophoretic mobility pattern in agarose gel. Ten aliquot portions of pBR322 DNA (15 ng) were dissolved in an appropriate volume of HEPES buffer solution (4 mM HEPES pH = 7.4, 5 mM KCl and 2 mM $MgCl_2$, Milli-Q water). Four of these portions were mixed with the required amount of previously dephosphorylated amifostine and another four with the required amount of amoxicilline corresponding to a molar concentration of ri = 0.5. A solution of cisplatin in a HEPES buffer was also prepared. The appropriate volume of this solution corresponding to a molar ratio DNA/cisplatin 0.5 was added to one of each sample containing WR-1065 and amoxicilline respectively. One aliquot was kept with the pBR322 DNA alone and the equivalent amount of cisplatin was added to other one. All the samples were incubated at 37 °C. After 1 h, the same amount of the cisplatin solution was added to one of each sample containing WR-1065 and amoxicilline, respectively which continued incubating at 37 °C. After a second hour, the same operation was realised adding the appropriate volume of the cisplatin solution to another pair of samples containing WR-1065 and amoxicilline respectively. The incubation of all the samples continued at 37 °C until a total time of 5 h.

2.3. Sample preparation for atomic force microscopy

Samples were prepared by placing a drop (3 μl) of DNA solution or DNA adducts solution onto peeled green mica (Ashville-Schoonmaker Mica Co., Newport New, VA). After adsorption for five min at room

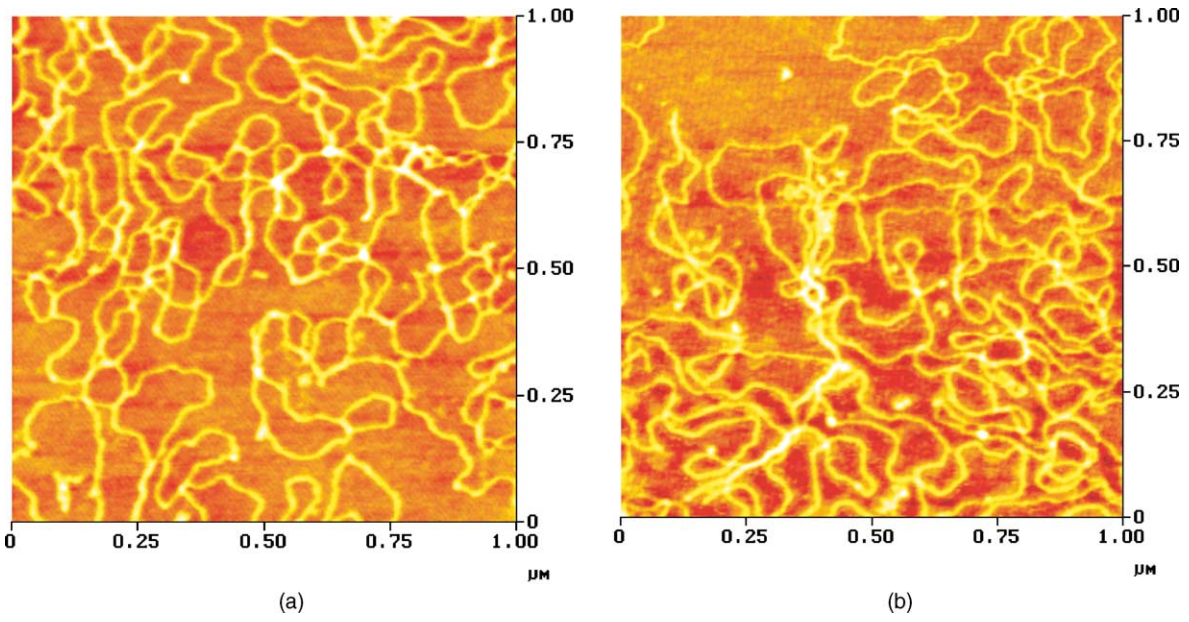

Fig. 2. TMAFM images in dry of pBR322 plasmid DNA in HEPES buffer at concentration of 0.5 ng/ml adsorbed on peeled mica corresponding to (a) free pBR322 plasmid DNA; (b) pBR322 plasmid DNA incubated for 5 h at 37 °C with cisplatin.

temperature, the samples were rinsed for 10 s in a jet of deionized water of 18 MWcm^{-1} from a Milli-Q water purification system directed onto the surface with a squeeze bottle. The samples were blown dry with compressed argon over silica gel and then imaged in the AFM.

2.4. Imaging by atomic force microscopy

The samples were imaged in a Nanoscope III Multimode AFM (Digital Instrumentals Inc., Santa Barbara, CA) operating in tapping mode in air at a scan rate of 1–3 Hz. The AFM probes were 125 mm-long monocrystalline silicon cantilever with integrated conical shaped Si tips (Nanosensors GmbH, Germany) with an average resonance frequency $f_o = 330$ kHz and spring constant $K = 50$ N/m. The cantilever is rectangular and the tip radius given by the supplier is 10 nm, with a cone angle of 35° and a high aspect ratio. In general, the images were obtained at room temperature ($T = 23 \pm 2$ °C) and the relative humidity (RH) was typically 55%. Four different samples of each reaction were imaged in several places and several times in order to obtain reliable measurements.

3. Results and discussion

A typical AFM image of relaxed pBR322 is shown in Fig. 2a. Most of the molecules are relaxed circular DNA distributed on the mica surface. The modifications caused in the DNA pBR322 by cisplatin can

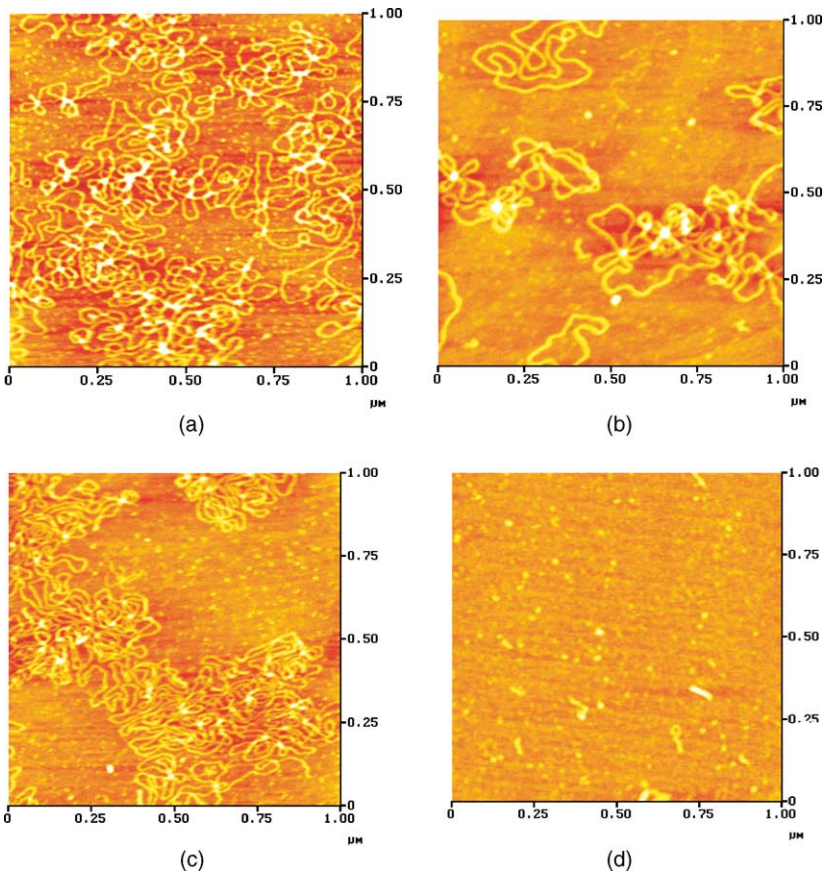

Fig. 3. TMAFM images in dry of pBR322 plasmid DNA in HEPES buffer at concentration of 0.5 ng/ml adsorbed on peeled mica: (a) incubated for 5 h at 37 °C with WR-1065; (b) the same DNA incubated simultaneously with cisplatin and WR-1065 for 5 h at 37 °C; (c) the same DNA incubated with only WR-1065 at 37 °C with addition of cisplatin after 1 h and incubated for 4 h more; (d) the same DNA incubated with WR-1065 at 37 °C with addition of cisplatin after 2 h and incubated for 3 h more.

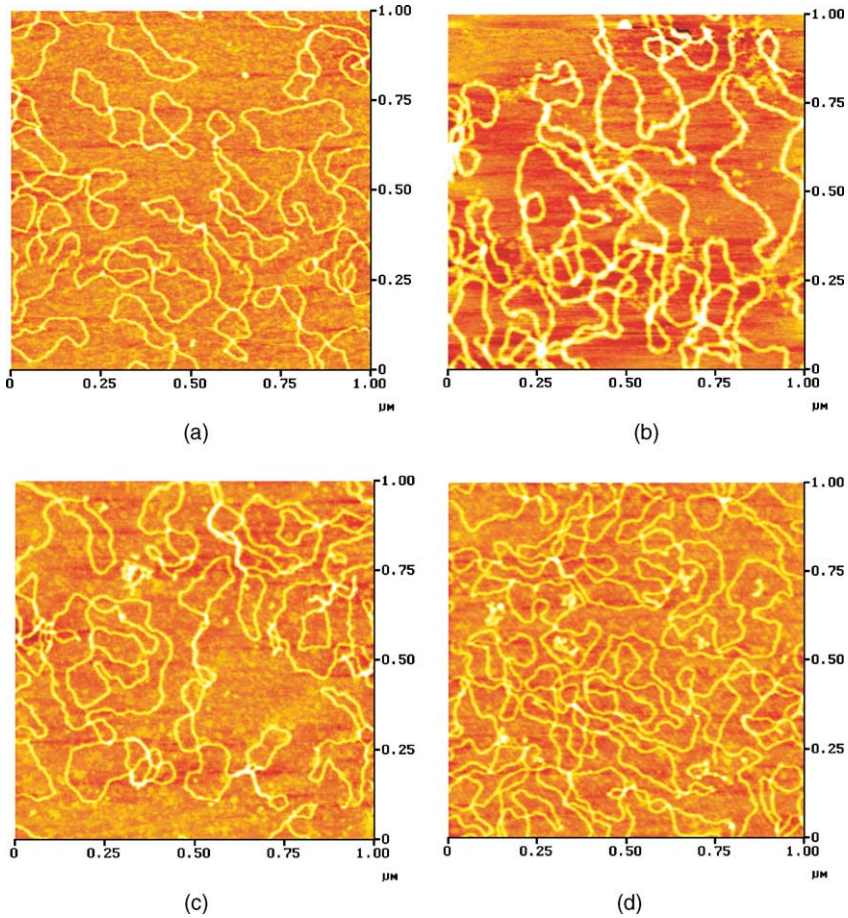

Fig. 4. TMAFM images in dry of pBR322 plasmid DNA in HEPES buffer at concentration of 0.5 ng/ml adsorbed on peeled mica: (a) incubated for 5 h at 37 °C with amoxicilline; (b) the same DNA incubated simultaneously with cisplatin and amoxicilline for 5 h at 37 °C; (c) the same DNA incubated with only amoxicilline at 37 °C with addition of cisplatin after 1 h and incubated for 4 h more; (d) the same DNA incubated with amoxicilline at 37 °C with addition of cisplatin after 2 h and incubated for 3 h more.

be observed in Fig. 2b. Images corresponding to the two different series of samples containing dephosphorilated amifostine (WR-1065) and amoxicilline after incubation at 37 °C are presented in Figs. 3a–d and 4a–d, respectively. Cisplatin mainly produces supercoiling of the DNA molecules and kinks in the double helix, but interconnection between molecules is not observed. This effect had previously been observed in linear DNA fragments (Onoa et al., 1997) and in pBR322 plasmid DNA (Cervantes et al., 1998; Onoa and Moreno, 2002). In Fig. 3a, the image shows the high aggregation of the pBR322 plasmid DNA caused by the addition of WR-1065. It is also possible to observe some ring-like structures connected by two or more molecules in a few areas. The simultaneous addition of cisplatin with dephosphorilated amifostine (WR-1065) produced the modifications observed in the Fig. 3b. The effect of the cisplatin is visually predominant over that of the WR-1065, but some degree of aggregation and connection between different molecules due to the dephosphorilated amifostine can also be distinguished. However, retarded addition of cisplatin (1 h after), Fig. 3c, seems to indicate that the action of the WR-1065 along time protects DNA against the strong effect of cisplatin and it is capable of modulate its action. The addition of cisplatin over adducts DNA/WR-1065 incubated 2 h at 37 °C and incubated at the same temperature for 2 h more pro-

duces an unexpected effect. In the Fig. 3d it can be observed the small spheres resulting from the DNA. This experiment was repeated several times in the same conditions and the images obtained were always identical. The effect of the amifostine as protector of the cisplatin action seems be optimised when amifostine is administered 1 h before that cisplatin.

These results are in good agreement with clinical and kinetic studies. In fact, the previous administration of amifostine in the cisplatin treatment (Korst et al., 1997, and Van der Vijgh and Korst, 1996) agrees with the kinetic studies realised. The action of cisplatin is faster than that of amifostine, and to avoid side effects such as nephrotoxicity, ototoxicity and others, it is necessary to administrate the protector amifostine before the administration of the drug cisplatin.

In Fig. 4a it can be observed that the amoxicilline produces the opposite effect that amifostine and aggregation and compactation decreases in some areas. In spite of the fact that the concentration of the samples was the same in all the cases, the amount of sample stuck on the mica surface during the preparation of the samples is evidently different. The image corresponding to the incubation at 37 °C for 5 h of pBR322 with amoxicilline and cisplatin is shown in Fig. 4b. Besides the predominant effect of supercoiling due to the cisplatin, aggregation can be also appreciated indicating interaction between DNA molecules through hydrogen bonds with amoxicilline molecules. Thus, the effects of both molecules, cisplatin and amoxicilline can be simultaneously observed.

In Fig. 4c and d, the behavior of the amoxicilline–cisplatin system can be observed when the time of the incubation of cisplatin is modified. After 1 h of incubation of DNA with amoxicilline at the same conditions of temperature, the same amount of cisplatin was added and the incubation continued up to complete the 5 h. The image obtained, Fig. 4c, shows that a lower number of molecules of DNA are supercoiled. In the image corresponding to a sample prepared by addition of the same amount of cisplatin to the DNA-amoxicilline adduct but now incubated for 2 h and as long as 5 h, Fig. 4d, the number of molecules of pBR322 supercoiled has decreased but the compactation has increased. Amoxicilline seems to protect the DNA from the action of cisplatin with independence of the time of addition of this drug after preparation of the DNA–amoxicilline adducts.

The amoxicilline seemed also to control the action of cisplatin on the cells, but the mechanisms of action on DNA of both molecules are probably different. The results suggest that amoxicilline or related antibiotics could be assayed as possible protecting agents in administration of cisplatin.

4. Conclusions

AFM can provide valuable information on the interaction of certain drugs with DNA. Significant changes in the topological form of relaxed pBR322 DNA plasmid can be observed by atomic force microscopy. Changes in the super-helical of the circular DNA are easily detected. Both molecules, dephosphorilated amifostine (WR1065) and amoxicilline seem to control the strong direct action of cisplatin on DNA. This effect can justify the role as protecting agent of amifostine on side effects caused by cisplatin and can open new possibilities to other agents as amoxicilline or to other similar S-containing molecules. Kinetic and AFM studies with other possible biological protecting agents are in progress.

Acknowledgements

We are grateful to CICYT, reference PB-98-1236 for financial support.

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