Cancer Chemother Pharmacol (1995) 35: 501–505

ORIGINAL ARTICLE

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Direct evaluation of intracellular accumulation of free and polymer-bound anthracyclines

Received: 24 March 1994 / Accepted: 3 October 1994

Abstract Nanoparticulate carriers of anthracyclines are being developed with the aim of improving the pharmacokinetic or pharmacodynamic behavior of these drugs. To understand how the drug reaches its nuclear targets, we have developed two methods that allow the quantification of the interaction between an anthracycline and cellular DNA: (1) by direct evaluation of the quenching of anthracycline fluorescence due to the intercalation of the drug into DNA and (2) by the measurement of Hoechst 33258 fluorescence associated with its displacement from DNA-binding sites for which it competes with the anthracycline. We show that the intracellular accumulation and DNA binding of doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres (dox-NS) and of daunorubicin bound to polyglutamic acid are reduced by 30%–40% in comparison with those obtained for free doxorubicin (dox) and daunorubicin, respectively. The results obtained with dox or NS-dox are not modified by prior incubation with either of these compounds. The two methods yielded similar results, and we conclude that either technique is applicable to the evaluation of the interaction of carrier-bound anthracyclines with cellular DNA.

Key words Anthracyclines · Drug carriers · Fluorescence spectroscopy

Introduction

Several forms of nanoparticulate carriers of anticancer drugs are presently under development. Attempts have focused on selecting carriers capable of modifying the pharmacokinetics of these drugs so as to obtain sustained plasma concentrations. In the case of anthracyclines, liposomes [9], DNA complexes [10], methacrylamide copolymers [6], and polyisoalkylcyanoacrylate nanospheres [5] have been developed. It is also expected that specific cell membrane targets can be exploited to modifiy the pharmacodynamics of anthracycline carrier complexes. For example, conjugation with transferrin or antibodies can take advantage of the presence of a high concentration of transferrin receptors or specific antigens on the membranes of tumor cells [8, 18]. Encapsulation can also help to overcome multidrug resistance, as has recently been shown with doxorubicin embedded in polyisohexylcyanoacrylate nanospheres [2] or liposomes [13].

The question as to whether the same number of anthracycline molecules can reach their target when the drug is presented free or bound to a polymer has not, however, been addressed. To evaluate this phenomenon, it is important to study the intracellular accumulation and DNA binding of the anthracycline, preferably in living cells. A new approach appropriate for such determinations has recently been developed by several authors, including ourselves [3, 14, 17], which consists of the measurement of the quenching of drug fluorescence following direct exposure of the cells to the anthracycline. When direct spectrofluorometry of anthracyclines is impossible because of low fluorescence of the drug complexes, another approach using DNA-specific fluorescent dyes can be used [4]; such dyes yield fluorescent complexes when they intercalate into DNA, and the competition between dye and anthracycline can be used to quantify anthracycline binding to DNA. Both approaches enable the study of the interaction of anthracycline with cells.

The purpose of this investigation was to show the potential of such approaches in the study of two anthracycline-polymer complexes: daunorubicin bound to polyglutamic acid and doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres.

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Materials and methods

Cell culture

Doxorubicin-sensitive rat glioblastoma cells (C6 clone) and a 6-fold doxorubicin-resistant variant selected by prolonged culture in the presence of 1 ng doxorubicin/ml medium were grown in monolayer cultures as described elsewhere [16]. Similarly, the human erythroleukemia K562 cell line and its doxorubicin-resistant variant were routinely grown in suspension cultures [1]. Human ovarian carcinoma cells (CaOv) were grown in medium 199 with 10% calf serum at 37 °C in glass vials. The cells were replated every week and the medium was changed every 2–3 days. Mouse thymocytes were obtained as previously described [4]. All cell types were studied as suspension of $1-2 \times 10^6$ cells/ml in buffered saline containing 5 m*M* glucose. Cell counts were performed either on an automatic hemocytometer (Coulter ZM) or using a microscope and a Goryaev chamber.

Drugs and chemicals

Doxorubicin hydrochloride was supplied by Laboratoire Farmitalia (Rueil-Malmaison, France); polyisohexylcyanoacrylate nanospheres, either free or containing encapsulated doxorubicin, were obtained from Dr. P. Couvreur (Chatenay-Malabry, France) and had been prepared as previously described [2]. Daunorubicin hydrochloride was obtained from Ferane (Russia); polyglutamic acid and polyglutamic acid-bound daunorubicin were supplied by the Laboratory of Chemical Synthesis (Russia). Experiments were performed with 10^{-6} *M* free doxorubicin or daunorubicin and with polymer-bound doxorubicin or daunorubicin at the same molar concentration. Blank polyisohexylcyanoacrylate nanospheres were also used at a concentration equal to that achieved when they are bound to 10^{-6} *M* doxorubicin. DNA-specific fluorescent dye Hoechst 33258 was obtained from Aldrich-Chemie. All other reagents were of the highest quality available.

Drug intracellular accumulation

Two methods were used to evaluate simultaneously the intracellular accumulation and DNA binding of anthracyclines. The first method is based on the significant fluorescence quenching that occurs when anthracyclines intercalate between DNA base pairs. Anthracycline accumulation and DNA binding were assessed by measurement of the fluorescence of a cell suspension in the cuvette of a spectrofluorometer upon the addition of an anthracycline [3, 14, 17]. The second method, developed by Bogush et al. [4], takes advantage of the intense fluorescence of Hoechst 33258 when it is complexed with DNA. The addition of other DNA-binding agents causes a reduction in this fluorescence, presumably because of competition for binding sites. Fluorescence was monitored with Hitachi M850 or Jobin-Y von JY3 spectrofluorometers in quartz cuvettes of 1×1 cm under continuous stirring and at a constant temperature of 37 °C. Experiments were initiated by the addition of the drug at a final concentration of 10^{-6} *M* to the cell suspension. Preliminary experiments had shown that cell uptake was linear at these concentrations. Excitation and emission wavelengths, respectively were, set at 480 and 590 nm for doxorubicin, at 470 and 550 nm for daunorubicin, and at 347 and 454 nm for Hoechst 33258. We verified that nonspecific adsorption of anthracyclines to the cuvettes was always less than 5%.

We confirmed that the fluorescence quenching of anthracyclines in cells is largely due to intercalation into DNA by the following method [7]. First, we determined the lowest concentration of Hoechst 33258 that yielded a near-maximal response in fluorescence for a given cell-suspension density; that is, the lowest amount required for saturation of cellular DNA-binding sites. Solutions of DNA were prepared and incubated with this concentration of dye, and the DNA concentration that produced the same fluorescence as that obtained with cells was determined. Assuming that DNA at this concentration contained the same number of binding sites as the cell suspension, we then compared the quenching of doxorubicin fluorescence by these two preparations in the absence of Hoechst 33258. Given that the quenching was nearly identical (less than 10% difference), we conclude that the quenching of doxorubicin fluorescence in cells is mostly due to interaction with DNA.

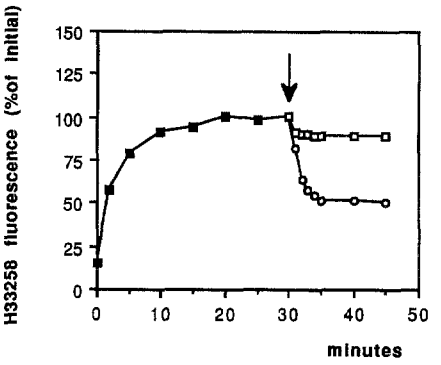


Fig. 1 Effect of daunorubicin and polyglutamic acid-bound daunorubicin on Hoechst 33258 fluorescence in CaOv cells. Cell suspensions (10^6 cells/ml) were incubated with 2×10^{-6} *M* Hoechst 33258 for 30 min (-■-); this was followed by the addition of 10^{-6} *M* daunorubicin (-○-) or polyglutamic acid-bound daunorubicin (-□-), and the incubation was continued for another 25 min. Three independent experiments were performed in triplicate, with less than 5% (within experiment) or 10% (between experiments) coefficients of variation. The results of a typical experiment are presented

Results

Daunorubicin, either free or bound to polyglutamic acid, was studied in a first series of experiments. We had previously shown that the fluorescence of the polymer-bound drug was approximately 10% of that of the free drug, which is not sufficient for a sensitive evaluation of the interaction of drug with DNA by the direct method. It was therefore necessary to study drug uptake and DNA binding by competition with Hoechst 33258. Figure 1 presents the results obtained in a typical experiment on CaOv cells. In the first 30 min, Hoechst 33258 interaction with DNA resulted in an important increase in fluorescence that remained stable for several hours; anthracycline addition was followed by a decrease in fluorescence that became maximal within 10 min and remained stable thereafter. Free daunorubicin (10^{-6} *M*) provided a 50% decrease in Hoechst 33258 fluorescence, whereas the same molar concentration of polyglutamic acid-bound daunorubicin caused only a 10% decrease in fluorescence.

In a second series of experiments, we compared the uptake and DNA binding of doxorubicin, either free or encapsulated in polyisohexylcyanoacrylate nanospheres. The fluorescence of encapsulated doxorubicin was about 50% of that of free drug. Drug fluorescence quenching could therefore be studied directly. Figure 2 shows the results of typical experiments with various types of sensitive and doxorubicin-resistant tumor cells and with murine thymocytes. The quenching of fluorescence decreased rapidly in an exponential-type fashion and reached a stable equilibrium after 10 min of incubation. In each case, there

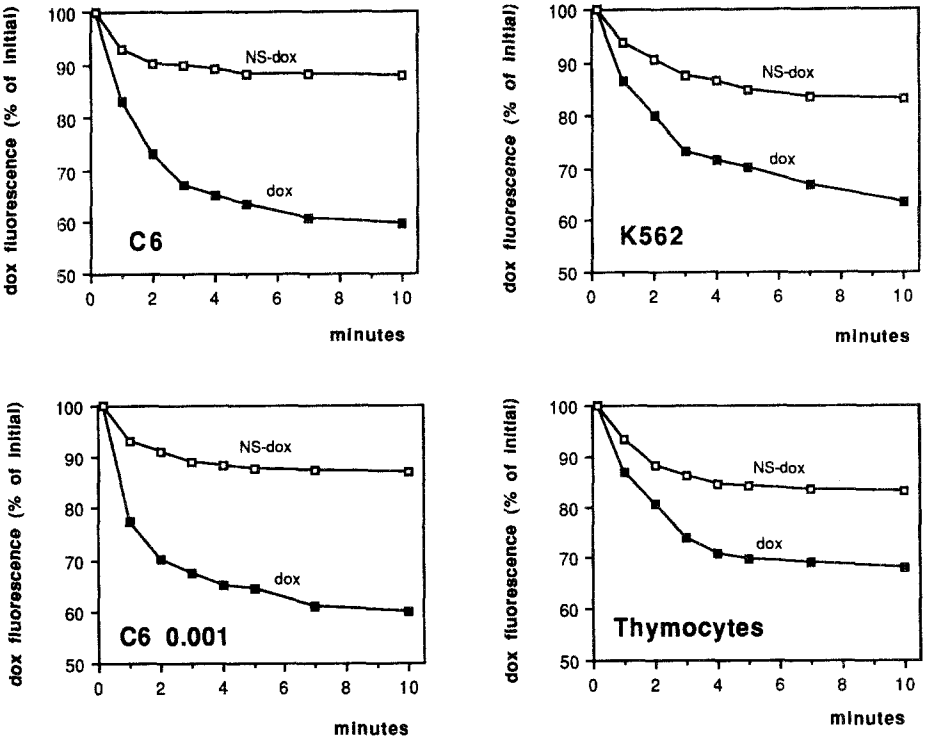
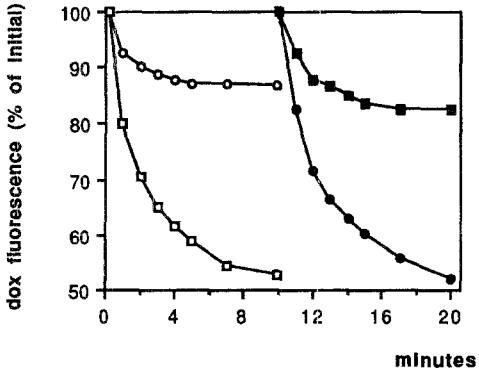


Fig. 2 Intracellular accumulation and DNA binding of free doxorubicin and doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres in several cell types. Cell suspensions (1.5×10^6 cells/ml) were incubated with either 2×10^{-6} *M* free doxorubicin (*dox*, -■-) or an equimolar amount of doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres (*NS-dox*, -□-) for 15 min. Three independent experiments were performed in triplicate, with less than 5% (within experiment) or 10% (between experiments) coefficients of variation. The results of a typical experiment are presented

was a significant difference between the results obtained with free doxorubicin and those achieved with nanoparticulate doxorubicin. A 30%–40% rate of fluorescence quenching was seen with free doxorubicin, whereas a quenching rate of only 10%–15% was seen when nanoparticulate doxorubicin was incubated at the same concentration with the same number of cells. The same experiment could be performed several times in a given cell suspension and the kinetics of the fluorescence quenching remained quantitatively the same. This enabled the comparison of two formulations to be made in each suspension studied. Figure 3 presents the results of successive addition of nanoparticulate doxorubicin after initial incubation with doxorubicin and of free doxorubicin after initial incubation with nanoparticulate doxorubicin. Prior incubation did not influence the fluorescence quenching observed during the second exposure, which remained in the ranges of 30%–40% for free doxorubicin and 10%–15% for nanoparticulate doxorubicin. The addition of blank nanospheres with free doxorubicin to the cell suspensions gave results similar to those obtained with free doxorubicin alone and no interference with the blank nanospheres was observed.

Fig. 3 Effects of dox and NS-dox preincubations on NS-dox and dox accumulation and DNA binding in sensitive C6 tumor cells. Cell suspensions (2×10^6 cells/ml) were incubated with 10^{-6} *M* dox (-□-) or NS-dox (-○-) for 10 min. Then, 10^{-6} *M* NS-dox (-■-) or free dox (-●-) was added and incubation was resumed for 10 min. Three independent experiments were performed in triplicate, with less than 5% (within experiment) or 10% (between experiments) coefficients of variation. The results of a typical experiment are presented



In a third set of experiments, we tried to evaluate the potential use of Hoechst 33258 for measuring doxorubicin accumulation and DNA binding in CaOv cells incubated with free doxorubicin or doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres. We compared these results with those obtained by the direct assessment of anthracycline fluorescence quenching. Both methods gave similar results (Fig. 4); doxorubicin accumulation and DNA binding was 1.5–1.8 times lower when the drug was incubated as nanospheres than when it was used as free drug. However, it should be mentioned that nanospheres, either blank or loaded with doxorubicin or accompanying free doxorubicin, had an influence on Hoechst 33258 basal fluorescence; the addition of nanospheres at concentration equivalent to 10^{-6} *M* doxorubicin when loaded resulted in a 2-fold rise in the basal fluorescence of the dye. It appears, therefore, that this method might be inaccurate for evaluating the cellular pharmacology of doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres; direct evaluation of fluorescence quenching seems to be a better method for such evaluations.

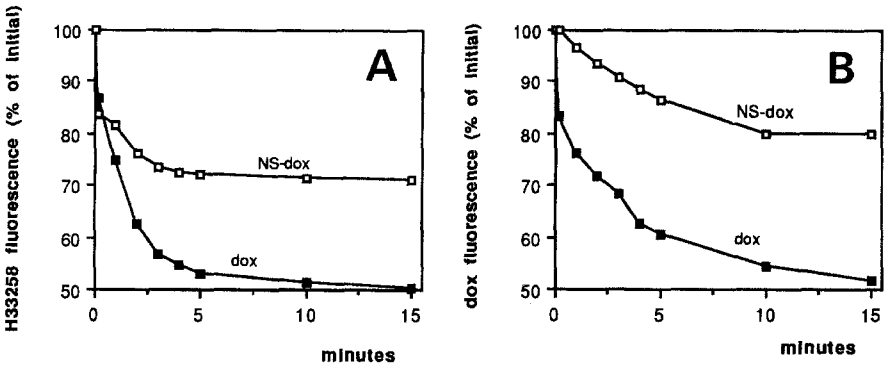


Fig. 4A, B Intracellular accumulation and DNA binding of dox and NS-dox in CaOv cells as measured by two different methods. **A** Cell suspensions (10^6 cells/ml) were incubated with 10^{-6} *M* Hoechst 33258 for 30 min (not shown); then, 10^{-6} *M* free dox (-■-) or NS-dox (-□-) was added to the cells and the incubation was resumed for 15 min. **B** Cell suspensions (10^6 cells/ml) were incubated with 10^{-6} *M* dox (-■-) or NS-dox (-□-) for 15 min. Three independent experiments were performed in triplicate, with less than 5% (within experiment) or 10% (between experiments) coefficients of variation. The results of a typical experiment are presented

Discussion

Anthracyclines represent one of the most powerful classes of anticancer drugs. Cardiac toxicity and occurrence of tumor-cell resistance are the two major problems encountered with these drugs, and these have stimulated important research efforts aimed at the discovery of new forms of administration, which would result in improved drug distribution (pharmacokinetics) and greater efficacy against drug targets (pharmacodynamics). Polymer-bound anthracyclines have been developed with these goals in mind, and a number of different vehicles have been proposed (for a review, see [15]). The cellular pharmacology of these complexes remains unclear, and the way the drug reaches its targets is far from being fully understood. The question as to whether the polymer enters the cell and the nucleus where the drug acts is of critical importance. The distribution of drug into the membrane, cytosol, biological macromolecules, and polymer is especially complex and a variety of techniques have to be applied to provide clues for the understanding of these processes. Methods used to evaluate total drug accumulation cannot distinguish between DNA-bound molecules and those sequestered in other cell compartments. The use of fluorescence techniques such as the ones we propose is therefore of great interest in studies of the pharmacology of polymer-bound anthracyclines in living cells.

We demonstrated in this study that the cellular DNA binding of two polymer-bound anthracyclines was decreased significantly in the same proportions in all of the cell models studied. Both techniques (direct fluorescence quenching of the drug or competition with a DNA-specific dye) gave similar results when tested in the same system. A significant reduction in total doxorubicin accumulation when the drug is applied in particulate form has previously been shown by Bennis et al. [2] in the same cell system. Herein we confirm these results and also provide the important information that a reduced number of DNA sites are reached when doxorubicin is embedded in nanospheres. Since the two formulations are equivalent in terms of cytotoxicity against C6 cells, this means that the absolute number of molecules that have reached their DNA target within 10 min is not a good indicator of cytotoxicity, in contrast to what has been claimed earlier [11].

We could not show a difference in doxorubicin accumulation and DNA binding between C6 0.001 cells, which present a relatively low degree of resistance (about 6-fold) to doxorubicin and sensitive C6 cells. This was rather surprising, since assays of total drug have consistently revealed a decreased accumulation, as would be expected in multidrug-resistant cells [2, 12]. The disparate result we obtained might be due to an insufficient sensitivity of our method; however, we think that it is likely to be due to a loss of P-glycoprotein efflux activity brought about by the handling of the cell monolayers during the preparation of the suspensions. In fact, we have shown in other experiments (not reported herein) that leukemic K562 resistant cells, which grow in suspension, exhibit via the direct fluorescence technique an important decrease in anthracycline accumulation and DNA binding. Work is in progress to obtain similar results with cells growing in monolayers.

Acknowledgements T. Bogush was the recipient of a travel grant from the Union Internationale Contre le Cancer. She is grateful to all the people working in the laboratory of J. Robert for their help during her stay, especially Drs. E. de Tinguy and L. Rivory and Mrs. F. Denois. This work was supported by the Russian Foundation of Basic Research (grant 11676-a) and by the International Soros Foundation (grant M3A000).

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