Biochemical Pharmacology, Vol. 45, No. 3, pp. 659–665, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

ACCUMULATION OF DEGRADATION PRODUCTS OF DOXORUBICIN AND PIRARUBICIN FORMED IN CELL CULTURE MEDIUM WITHIN SENSITIVE AND RESISTANT CELLS

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(*Received* 30 *April* 1992; *accepted* 27 *October* 1992)

Abstract—Quantitative study of doxorubicin (Adriamycin®), pirarubicin (4′-*o*-tetrahypyranyladriamycin) and daunorubicin in the nucleus of living cells was performed using microspectrofluorometry. As for the cytotoxic assays, drug-sensitive and drug-resistant K562 cells were incubated for 3 days with concentrations of drug ranging from 4 nM to 1 mM. When drug-sensitive cells were incubated with pirarubicin, the spectrum recorded from inside the nucleus was characteristic of anthracycline intercalated between the base pairs in the nucleus. However, when drug-sensitive cells were incubated with doxorubicin and drug-resistant cells with pirarubicin or doxorubicin, a new fluorescent spectrum was obtained which was due to 7,8-dehydro-9,10-desacetyldoxorubicinone, a pirarubicin and doxorubicin degradation product that is formed in the medium. This compound which is highly lipophilic is taken up rapidly into both sensitive and resistant cells.

Doxorubicin (Adriamycin®), pirarubicin (4′-*o*-tetrahypyranyladriamycin) and daunorubicin are widely used clinically for cancer chemotherapy. Their effectiveness is limited by the development as with other antitumor compounds, of multidrug resistance (MDR†) [1, 2]. One of the most important features of MDR is the over-expression of an integral plasma membrane glycoprotein termed P-glycoprotein [3–6]. This protein is responsible for active efflux of the drug from the cells and decreasing the intracellular drug concentration to a level which is not sufficient to inhibit cell proliferation [3, 7].

We have recently developed a simple spectrofluorometric method to follow the uptake of anthracycline by sensitive and resistant cells [8–12]. This method, which requires only a classical spectrofluorometer, is very useful when drug concentrations range from 10^{-7} to 10^{-5} M. However, it is not applicable to low drug concentrations such as those used for cytotoxic assays.

In order to determine the relationship between intracellular drug concentration and degree of resistance, we wished to measure drug concentration inside the cells, under the same conditions as those used for cytotoxic assays. For this purpose we used a microspectrofluorometer which allows the recording of the fluorescence spectrum from a small volume inside one cell [13, 14]. We were surprised to observe, when cells were incubated with doxorubicin or pirarubicin, the presence inside the cell of an anthracycline derivative exhibiting a spectrum different from that of the parent drug. The amount of this derivative present inside the cell increased as a function of the time of incubation. We show that this derivative is recovered inside the cells when they are incubated with doxorubicin or pirarubicin but not with daunorubicin and that it is a degradation product of doxorubicin or pirarubicin which is formed in the medium. It has been identified as 7,8-dehydro-9,10-desacetyldoxorubicinone (hereafter named D*). This product is very lipophilic, enters the cells very rapidly and accumulates inside the nuclei of drug-sensitive as well as drug-resistant cells.

MATERIALS AND METHODS

Drugs and chemicals. Purified doxorubicin and pirarubicin were kindly provided by Laboratoire Roger Bellon (France); daunorubicin was from Laboratoire Rhône-Poulenc (France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11{,}500\ M^{-1}cm^{-1}$. As anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just prior to use. All other reagents were of the highest quality available, and deionized double-distilled water was used throughout the experiments. Unless otherwise stated buffer solutions were 9.5 mM Hepes buffer (plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, with 5 mM glucose) at pH 7.2.

Absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer and macrofluorescence spectra on a Jobin Yvon, JY3CS spectrofluorometer.

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† Abbreviations: MDR, multidrug resistance; D*, 7,8-dehydro-9,10-desacetyldoxorubicinone.

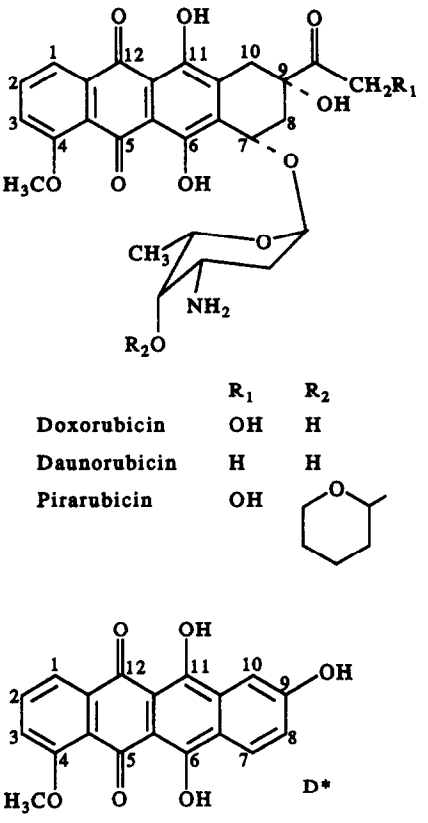
High molecular mass calf thymus DNA was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and dissolved in 0.1 M NaCl, 0.05 M Hepes buffer at pH 7.2 for 3 hr under vigorous stirring. A nucleotide absorption coefficient of 6600 $M^{-1}cm^{-1}$ was used to calculate DNA concentrations from absorbance at 260 nm.

Doxorubicin, pirarubicin and their degradation products were separated using ascending TLC on Stratochrom plates (RS 0.25 mm, 5 × 20; Carlo Erba, Italy). Two different developing solutions were used: (i) 90:10:1 (by vol.) chloroform:methanol:6 N NH_3 with R_f equal to 0.20 (doxorubicin), 0.47 (pirarubicin), 0.56 (D*), 0.78 (doxorubicinone); (ii) 40:10:0.1 (by vol.) chloroform:methanol:glacial acetic acid with R_f equal to 0.54 (doxorubicin), 0.84 (pirarubicin), 0.90 (D*), 0.95 (doxorubicinone). All products were identified by co-spotting of an authentic sample.

Cell lines and cultures. K562 cells were a gift of Dr Tapiero (Departement de Pharmacologie Cellulaire, ICIG, 94800 Villejuif, France). They were grown in RPMI 1640 medium (Sigma), containing 10% heat-inactivated fetal calf serum (Sigma), 2 mM L-glutamine (Sigma), streptomycin (0.1 mg/mL), penicillin (100 U/mL), 23 mM sodium bicarbonate and 10 mM Hepes (pH 7.3) at 37° in a humidified atmosphere with 5% CO_2. Cells were grown either in the absence or presence of anthracycline. In the absence of drug, cultures initiated at a density of 10^5 cells/mL grew exponentially to about 10^6 cells/mL in 3 days. IC_{50}, the drug concentration required to inhibit 50% of cell proliferation, was equal to 10 nM (S) and 300 nM (R) for doxorubicin, 4 nM (S) and 32 nM (R) for pirarubicin, 7 nM (S) and 166 nM (R) for daunorubicin where (S) and (R) stand for drug-sensitive and drug-resistant cells, respectively. *R*, the "resistance factor" obtained by dividing the IC_{50} of resistant cells by the IC_{50} of sensitive cells was equal to 30, 8 and 24 for doxorubicin, pirarubicin and daunorubicin, respectively. Cells were also grown in the presence of drug and 5 μM verapamil which alone, at this concentration, does not inhibit cell proliferation, but is well-known to reverse MDR. The antitumor properties of D* were also determined and ID_{50} was found to be higher than 700 nM. Cell viability was assessed by trypan blue exclusion, and cell counting was made with a Coulter counter.

Microspectrofluorescence measurements. The fluorescence emission spectra of doxorubicin, pirarubicin and daunorubicin exhibit bands at 560, 590 and 620 nm. When the molecules intercalate between the base pairs of DNA in the nucleus, strong quenching of the drug fluorescence is observed. The fluorescence of the DNA-bound drug is about 40 to 50 times lower than that of the free drug [11, 15]. However, it is possible, with the microspectrofluorometer to record the fluorescence spectrum of the drug in the nucleus.

Fluorescence emission spectra from intracellular microvolume were recorded with an UV-visible microspectrofluorometer prototype developed in our laboratory which has been extensively described elsewhere [13, 14]. Briefly, excitation was achieved by an Ar^+-laser tuned at the 488.0 nm line. A 100× objective and a luminous field diaphragm were used on the excitation path focusing the laser beam on a spot of less than 1 μm diameter. Fluorescence spectra were recorded in the region 500–750 nm on a 1024 diode intensified optical multichannel analyser (Princeton Instruments). In order to avoid side-effects such as cell death and photochemical effects, neutral density filters were used to minimize the intensity of the excitation beam. Typically, the light power was reduced to 0.1–0.5 μW on the illuminated spot. In order to control laser power and instrumental response, and to make possible quantitative comparison, a uranyl bar was used as a fluorescence standard. For microspectrofluorometric analysis an aliquot of culture medium containing cells was placed on a slide (Malassez). Measurements of the fluorescence of at least 10 nuclei in each sample were made for 15–20 min at 20–22°. Data were stored and processed on a 80286 IBM PS/2 microcomputer, using the Jobin-Yvon "Enhanced Prism" software.



	R_1	R_2
Doxorubicin	OH	H
Daunorubicin	H	H
Pirarubicin	OH	

Scheme 1.

RESULTS

The structures of the three anthracycline derivatives used in the present study are shown in Scheme 1.

In a first set of experiments, drug-sensitive and drug-resistant cells were grown, for 3 days, in the presence of doxorubicin or pirarubicin at

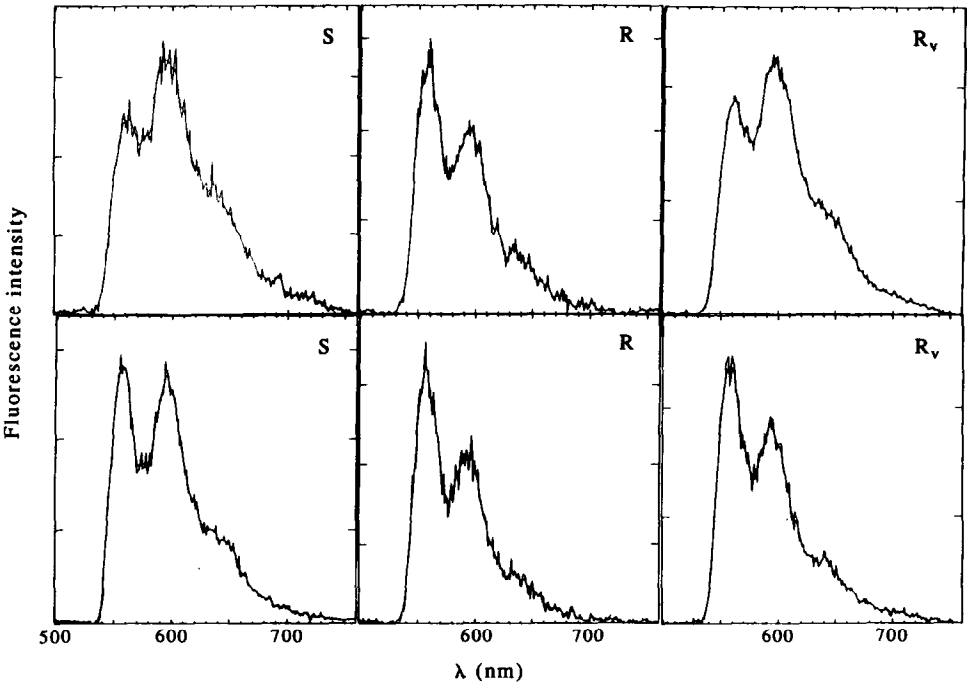


Fig. 1. Corrected fluorescence emission spectra of pirarubicin- and doxorubicin-treated K562 cell nucleus. Upper: 10^5 cells/mL were incubated for 3 days in the presence of 40 nM pirarubicin. Lower: 10^5 cells/mL were incubated for 3 days in the presence of 50 nM doxorubicin. S and R stand for drug-sensitive and drug-resistant cells, respectively. R_v stands for drug-resistant cells in the presence of 5 μM verapamil.

concentrations used for the cytotoxic assays. The pH of the medium was kept constant and equal to 7.3 ± 0.1 throughout all the experiments. We observed that, in the absence of Hepes in the culture medium, the pH was equal to 8 at $t = 0$ and decreased to 6.9 after 3 days of incubation. After 3 days, the cells were counted and the microspectrofluorescence spectrum of the drug inside the cell nuclei was recorded.

Intranuclear uptake of pirarubicin after 3 days incubation of cells with drug

Anthracycline-sensitive cells were incubated with pirarubicin at concentrations ranging from 1 to 40 nM ($IC_{50} = 4$ nM). Figure 1 shows the typical spectrum recorded from a microvolume inside the nucleus when cells were grown in the presence of 40 nM pirarubicin. Spectra have also been recorded at concentrations equal to 10 nM, but no reliable data could be obtained at lower concentrations. This spectrum is typical of that of an anthracycline molecule intercalated between DNA base pairs [15]: it exhibits a first band of lower intensity at 560 nm, a higher intensity band at 590 nm and a shoulder at 620 nm. The fluorescence yield of the DNA-bound drug is about 40 to 50 times lower than that of the free drug [11, 15]. In the following, the shape of the fluorescence spectrum will be characterized by the value of ρ, the ratio of the fluorescence intensity of the band at 560 nm to that at 590 nm. In the case of incubation of drug-sensitive cells with pirarubicin, the ratio was found equal to 0.78 ± 0.02. With drug-resistant cells, the results were different. The pirarubicin concentrations ranged from 10 to 500 nM ($IC_{50} = 32$ nM). Figure 1 shows the typical spectrum recorded from a microvolume inside the nucleus, when the drug-resistant cells were incubated with 40 nM pirarubicin. As can be seen, the shape of the spectrum is different, exhibiting higher intensity bands at lower wavelengths with $\rho = 1.30 \pm 0.05$. Reliable data have been obtained for the whole range of concentrations used. The fluorescence intensity was proportional to the concentration (Fig. 2).

Figure 1 also shows the spectrum recorded when drug-resistant cells were incubated with 40 nM pirarubicin and 5 μM verapamil, when the fluorescence spectrum was closely related to that obtained with drug-sensitive cells.

Intranuclear uptake of doxorubicin after 3 days incubation of cells with drug

Cells were incubated for 3 days with doxorubicin at concentrations ranging from 1 to 50 nM ($IC_{50} \sim 10$ nM) in the case of drug-sensitive cells, 50 to 10^3 nM ($IC_{50} \sim 280$ nM) in the case of drug-resistant cells and 5 to 400 nM in the case of drug-resistant cells in the presence of 5 μM verapamil.

Figure 1 shows the fluorescence spectrum recorded from the nucleus of drug-sensitive and drug-resistant cells, in the absence and in the presence of verapamil, when incubation was performed with 50 nM

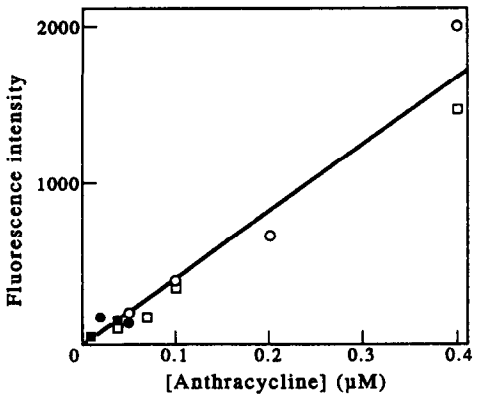


Fig. 2. Incubation of K562 cells with doxorubicin and pirarubicin. Drug-sensitive cells (filled symbols) and drug-resistant cells (open symbols) were incubated for 3 days in the presence of different concentrations of either doxorubicin (circles) or pirarubicin (squares). The fluorescence intensity at 590 nm recorded from the cell nucleus has been plotted as a function of the drug concentration. The fluorescence intensity of the uranyl bar, used as a fluorescence standard, was equal to 10^5.

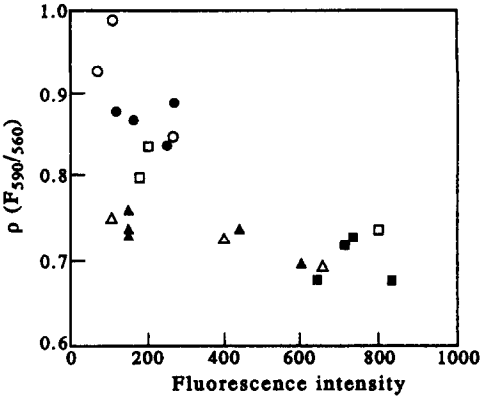


Fig. 3. Incubation of K562 cells with daunorubicin (triangles), doxorubicin (circles) and pirarubicin (squares). Cells (10^6/mL) were incubated for 2 hr in the presence of 1 μM drug. The cells were either drug-sensitive (filled symbols) or drug-resistant (open symbols). The fluorescence intensity has been recorded from the nucleus and the ratio ρ of the fluorescence intensity at 560 nm to that at 590 nm has been plotted as a function of the fluorescence intensity at 590 nm. The fluorescence intensity of the uranyl bar, used as a fluorescence standard, was equal to 2×10^4.

doxorubicin. As can be seen, the ratio ρ of the fluorescence intensity is, in all three situations, higher than 1. For drug-resistant cells several concentrations could be studied. The fluorescence intensity was proportional to the concentration used (Fig. 2).

Intranuclear uptake of drug after short incubation of cells with doxorubicin or pirarubicin which have been left for 3 days in the culture medium

In order to determine if the new fluorescence spectrum, with $\rho \sim 1.6$, could be assigned to a doxorubicin and pirarubicin degradation product, the following experiments were performed. Doxorubicin or pirarubicin (500 nM) was incubated in the culture medium at 37° for 3 days; the conditions were the same as those used for cell culture except that the cells were omitted. After 3 days, 10^6 cells/mL were added to this medium, incubated for about 5 min, and microspectrofluorescence spectrum was recorded at once. Spectra, similar to those observed when drug-resistant cells were grown for 3 days in the presence of the same concentration of drug (Fig. 2), was obtained ($\rho \sim 1$ and comparable fluorescence intensity). This strongly suggested that, as a function of time, a new derivative was formed in the medium. In addition, the uptake of this new derivative by drug-sensitive as well as drug-resistant cells is very fast. This was corroborated by the following measurements performed after a short incubation of the cells with drug.

Intranuclear uptake of doxorubicin, pirarubicin and daunorubicin after short incubation of drug with cells

In the following section we report data of experiments in which the time of incubation of 10^6 cells/mL with 1 μM drug was ranging from 10 min to 3 hr. In the case of the uptake of daunorubicin by cells, we never observed the appearance of a fluorescence spectrum exhibiting a ρ value higher than 0.78. We inferred that the degradation product, exhibiting the typical fluorescence spectrum which is formed when pirarubicin or doxorubicin are used, is not obtained with daunorubicin. However, a slight decrease of ρ value from 0.75 to 0.70 was observed when the fluorescence intensity recorded from the nucleus was increasing, i.e. when the intracellular drug concentration was increasing. The variation of ρ as a function of the fluorescence intensity is due to the fact that not only the intensity but also the shape of the fluorescence spectrum depends on the drug concentration (manuscript in preparation). The ρ values obtained in the case of the uptake of daunorubicin by drug-resistant cells compared to that obtained in the case of drug-sensitive cells are shown in Fig. 3.

When drug-sensitive cells were incubated with pirarubicin a steady state was reached within 30 min [11]. In that case ρ was 0.71 ± 0.03 and compared with the value obtained with daunorubicin. The ρ value remained constant during the following hours. When drug-resistant cells were incubated with pirarubicin, the steady state was also reached within 30 min, but the ρ value was higher being equal to 0.82 ± 0.02. However, when the incubation was performed in the presence of 5 μM verapamil, a ρ value comparable to that obtained with drug-sensitive cells was observed.

In the case of doxorubicin, the time required to reach the steady state was very long and we have previously determined that, under our experimental conditions, about 5 hr are required for sensitive and resistant cells [8, 11, 12]. The ρ values obtained after 3 hr of incubation were 0.96 ± 0.02 for drug-resistant

cells and 0.87 ± 0.02 for both drug-sensitive cells and drug-resistant cells in the presence of verapamil.

In order to determine whether the modification in the shape of the fluorescence spectrum, as characterized by the ρ value, is due to variation of the intranuclear drug concentration, or to the degradation product formation, the variation of ρ was plotted as a function of fluorescence intensity in Fig. 3. As can be seen, for comparable fluorescence intensity, ρ values follow the order:

$$\rho_{DOX}(R) > \rho_{DOX}(S) > \rho_{DOX}(R + \text{verapamil}) > \rho_{PIR}(R) > \rho_{DNR}(R, S) \sim \rho_{PIR}(S) \sim \rho_{PIR}(R + \text{verapamil})$$

where DOX = doxorubicin; PIR = pirarubicin; DNR = daunorubicin; and (S) and (R) stand for drug-sensitive and drug-resistant cells, respectively.

Doxorubicin and pirarubicin degradation product formation in Hepes buffer

The doxorubicin and pirarubicin degradation products formed in culture medium and in aqueous solution, at different pH values have been extensively studied by Beijnen *et al.* [16–19]. They have shown that, in the case of doxorubicin, at pH > 6, the main degradation product was D* (Scheme 1). The degradation reaction follows a pseudo-first-order kinetics. Using their data we have calculated that, at pH 7.3 and $t = 37°$, within 3 days, 60% of the parent molecule should have been degraded. The D* degradation product was isolated using the following procedure: 10^{-4} M doxorubicin was incubated for 3 days in Hepes buffer at pH 7.3 and 37° (the results were similar when phosphate or Tris buffer were used). After 3 days, it was arrested by TLC and revealed two compounds present in the solution. As the water solubility of D* is very low ($<10^{-5}$ M) the concentration [D], of the parent molecule remaining in the aqueous solution was determined spectroscopically. The difference between the initial concentration of doxorubicin and [D] yielded the D* concentration formed in the buffer. In order to recover quantitatively the D* formed, the solution was acidified and the D* product extracted by the addition of organic solvents (4:1 $CHCl_3$:MeOH v/v). The organic phase contained D* and a small amount of the parent molecule, the two compounds were separated by TLC with 90:10:1 (by vol.) chloroform:methanol:6 N NH_3. The spot with $R_f = 0.56$ corresponding to a known amount of D* was scraped off and eluted with 4:1 (v/v) chloroform:methanol. A D* solution of known concentration was thus obtained.

The absorption spectrum of D* in solution in chloroform:methanol (1:4, v/v) exhibited maxima at 542 nm ($\varepsilon_M = 10{,}000$), 505 ($\varepsilon_M = 11{,}500$) and 475 ($\varepsilon_M = 8000$). The fluorescence spectrum obtained through excitation at 480 nm exhibited bands at 560 and 595 nm. The ratio ρ of the fluorescence intensity at 560 nm to that at 595 nm was equal to 1.6.

The same procedure was used with pirarubicin and the same D* degradation product was obtained. No degradation product was detected with daunorubicin. Analogous data were obtained when the drugs were incubated in RPMI medium in the absence or presence of Hepes.

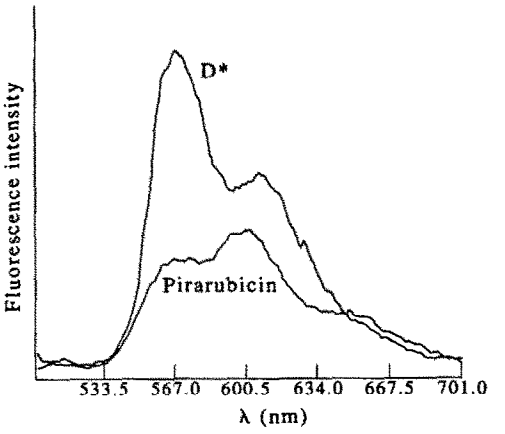


Fig. 4. Fluorescence spectrum of D* and pirarubicin in the presence of DNA. Experimental conditions: [D*] = 30 μM, [pirarubicin] = 10 μM, DNA [bp] = 1.1×10^3 M, Hepes buffer pH = 7.2, excitation wavelength 480 nm.

Comparison of the interaction of D and doxorubicin or pirarubicin with DNA*

Although D* has a water solubility lower than 10^{-5} M, an aqueous solution can be obtained by addition of a small amount of D* in dimethyl sulfoxide to water. In these conditions, the fluorescence emission spectrum is very weak with maxima at 565 and 592 nm ($\rho = 0.95$). The maximum of D* fluorescence intensity is about 1/450 of that of pirarubicin in the same conditions. It is well known that anthracycline derivative fluorescence is quenched through intercalation of the molecules between base pairs in DNA. The ratio of the fluorescence yield of doxorubicin bound to DNA and free doxorubicin is about 1/40 [15]. The opposite effect was observed with D*: through interaction with DNA its fluorescence spectrum was modified and the quantum yield enhanced. The ratio of the fluorescence quantum yield of D* bound to DNA and free in aqueous solution is about 20. The fluorescence spectra of D* in the presence of 1 mM DNA (bp) in Hepes buffer exhibited bands at 567 and 605 nm. Under the same conditions, the fluorescence spectrum of pirarubicin exhibited bands at 573 and 598 nm (Fig. 4). As can be seen, the quantum yield of D* bound to DNA compares to that of pirarubicin bound to DNA.

Uptake of D by drug-sensitive and drug-resistant cells*

When cells were incubated with D*, the emission spectrum recorded in the nucleus exhibited a high intensity band at 560 nm and a lower intensity band at 590 nm, ρ value being 1.6 ± 0.2. The D* uptake by cells was very fast: when 10^6 drug-resistant or drug-sensitive cells were incubated with 1 μM D*, the first measurement was performed within 3 min and no variation of the fluorescence intensity was

observed during the following hour, indicating that the steady state was reached within the first 3 min. In addition, the fluorescence intensity recorded from the nucleus was the same for drug-sensitive and drug-resistant cells. This suggests (i) D* is not extracted from the cells by P-glycoprotein, (ii) the MDR efflux system has a finite speed and that highly lipophilic compounds can overwhelm the efflux system since drug uptake occurs too rapidly for efflux to compete effectively. In a second experiment, pirarubicin was added to drug-resistant cells in the presence of D*. At steady state, the amount of pirarubicin in the nucleus was the same as when the incubation was performed in the absence of D*, strongly suggesting that D* is not able to compete with the original anthracycline and modulate its intracellular concentration.

DISCUSSION

The instability of anthracycline derivatives in aqueous solution and in cell culture media has been recognized by several investigators. Among them Beijnen *et al.* [16–19] have carefully identified the anthracycline degradation products that are formed either in acidic media, pH ~ 4, or slightly basic media, pH ~ 8, as well as those formed in cell culture media enriched with serum. They have clearly shown that, in the case of doxorubicin at pH higher than 6, the main degradation product is a pink-colored product in which full aromatization of the A ring system has occurred. It has been identified as D*. These authors have studied the degradation kinetics as a function of ionic strength, temperature, pH, drug concentration. They have shown that the degradation of doxorubicin follows pseudo-first-order kinetics at constant pH, temperature and ionic strength under the various pH conditions.

From these data we can estimate that in aqueous solution at pH 7.4, 37° and 0.1 M ionic strength, the half-life time of doxorubicin is about 18 hr and that after 2 hr and 3 days about 3 and 60% of doxorubicin has undergone degradation, respectively. In addition, they have observed that the 4′-congeners of doxorubicin, i.e. 4′-deoxydoxorubicin, 4′-*O*-methyl doxorubicin and 4′-epidoxorubicin all degrade with almost the same velocity.

Under our experimental conditions, i.e. doxorubicin or pirarubucin left for 3 days in the culture medium, we have observed that about 50% of the drug was degraded. This is in good agreement with the data of Beijnen *et al.* [16–19]. In both cases we obtained a major degradation product with absorption and fluorescence spectra similar to that reported by Beijnen *et al.* This means that pirarubicin, which is also a 4′-congener of doxorubicin, is degraded in the same way. In both cases D* is formed which has a very low antitumor activity.

Degradation of daunorubicin at pH ~ 8 requires very drastic conditions such as 4 hr at 100° when the major degradation product has been identified as 7,8,9,10-bis-anhydrodaunorubicinone [19] which exhibits absorption and fluorescence spectra which are distinct from the parent molecule. In cell culture media daunorubicin is also much more stable than doxorubicin and, under our experimental conditions, we never detected a fluorescence spectrum different from that of daunorubicin.

When doxorubicin or pirarubicin are left for 3 days at 37° in cell culture media about 50% of the drug is degraded to D*. When cells, either drug-sensitive or drug-resistant, are added to the medium the uptake of D* occurs at once and the amount of D* present in cell nuclei is the same, in both sensitive and resistant cells.

When drug-sensitive cells were incubated with pirarubicin the spectrum recorded from the nucleus, either after short incubation (1–3 hr) or long incubation (3 days), was always typical of that of the parent drug intercalated between base pairs in the nucleus ($\rho \sim 0.76$) indicating that no detectable D* product has been accumulated inside the nucleus. However, when drug-sensitive cells are incubated with doxorubicin the ρ value is 0.87 after 2 hr incubation and 1 after 3 days, indicating that both the parent drug and the degradation products are present inside the nucleus. The different findings obtained with the two drugs can be explained by the fact that their uptake by cells did not occur at the same rate. For pirarubicin the steady state is reached within about 30 min [8, 11, 12]. This means that once the drug has entered the cell and is intercalated between the base pairs in the nucleus it is protected from degradation. In the case of doxorubicin which enters the cell very slowly, some of the drug undergoes degradation before the steady state has been reached and D* enters the cell, explaining why a small amount of D* is found inside the cell after 2 hr ($\rho = 0.87$) and a large amount after 3 days. In the case of drug-resistant cells, the amount of D* in cells is high for both drugs and, from the fluorescence intensity, the amount can be seen to be roughly proportional to the initial concentration of drug used for incubation.

From the ρ value and using the relation $\rho = \rho_{D^*} f_{D^*} + \rho_D f_D$, it is possible to estimate the relative amount of D* and of the parent molecule present in the nucleus: in this relation, ρ is measured from the spectrum recorded in one cell nucleus, f_D and f_{D^*} are the molar fraction of D and D* present in the nucleus, respectively ($f_D + f_{D^*} = 1$). Thus, when 10^5 cells/mL were incubated for 3 days with 40 nM pirarubicin, we can estimate that in drug-sensitive cells 100% of the drug was present as the parent molecule ($\rho = 0.78$) but only 37% in drug-resistant cells ($\rho = 1.30$). When the cells were incubated under the same conditions with doxorubicin, 73% of the drug was present as the parent molecule ($\rho = 1$) in sensitive cells and only 22% in drug-resistant cells ($\rho = 1.42$).

D* is not only obtained under the conditions described above: we have recently shown that it is rapidly formed by interaction of doxorubicin and pirarubicin with Fe(III).†

In conclusion, we have shown that the doxorubicin and pirarubicin degradation product which is formed in culture media rapidly accumulates inside both

† Fiallo MML and Garnier-Suillerot A, Characterization and isolation of adriamycin and THP-adriamycin degradation product formed through interaction with iron, submitted.

sensitive and resistant cells. This data is of importance as far as it may confuse the results of biological *in vitro* assays in which cultured tumor cells are exposed to the drugs. D* is not extracted from the resistant cells by P-glycoprotein and thus not able to compete with the original parent anthracycline and modulate its intranuclear concentration.

Acknowledgement—This investigation was supported by Université Paris Nord, Pierre et Marie Curie, le Centre National de la Recherche Scientifique et l'Institut Curie.

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