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Selective analysis of antitumor drug interaction with living cancer cells as probed by surface-enhanced Raman spectroscopy

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Abstract. A new technique for the selective measurement of small amounts of antitumor drugs in the nucleus and cytoplasm of a living cancer cell, based on surface-enhanced Raman spectroscopy (SERS), is proposed. The ability to detect SERS signals from very dilute (up to 10^{-10} M) solutions of doxorubicin or adriamycin (DOX), and 4′O-tetrahydropyranyl-adriamycin (THP-ADM), as well as from their complexes with targets in vitro and in vivo, has been demonstrated. SERS spectra were obtained from a population as well as from single living erythroleukaemic K562 cells treated with DOX. The results of the measurements on the population of cells containing DOX in nuclei or in the cytoplasm are well correlated with the microscopic SERS measurements on the single cells treated with DOX, obtained by selectively recording signals from the living cell nucleus or from the cytoplasm. Possibilities for the application of this new technique in different aspects of cancer research are discussed.

Key words: Living cancer cell – Doxorubicin – Surface-enhanced Raman spectroscopy

Introduction

It has recently been demonstrated that the effect of very large (in some instances up to 10^9) enhancements of the Raman cross-section for molecules in the close vicinity of a metal surface has many applications in the study of water-soluble proteins, peptides and amino acids (Nabiev and Efremov 1983; Cotton 1988; Chumanov et al. 1990; Nabiev and Chumanov 1986), membrane-bound complexes (Abdulaev et al. 1987; Nabiev et al. 1985; Nabiev et al. 1990a), nucleic acids and their complexes with peptides and different low-molecular weight compounds (Nabiev et al. 1988; Koglin and Sequaris 1986) including antitumor drugs and their complexes with DNA in vitro (Smulevich and Feis 1986; Nonaka et al. 1990). This is only a very short list of biologically important molecules which have been studied by the new physical chemical technique of Surface-Enhanced Raman Spectroscopy (SERS).

It should be stressed that the optimal experimental conditions, which do not perturb the structure of the molecules under investigation, have been found in many biological applications of SERS (Nabiev et al. 1990a). The obvious prospects of this technique led us to examine SERS applications in structure-function investigations in living cells. This paper deals with investigations on some antitumor drugs, their complexes with DNA as well as the interaction of one of them – DOX – with living cancer cells. We selectively detected the signals from this drug incorporated in the cytoplasm or in the nucleus of the single living cell under the microscope as well as the signals for a population of living cells. The possibility of in vivo detection of signals from DNA in the nucleus of the living cancer cell has also been demonstrated.

Materials and methods

Drugs and chemicals

Stock solutions (100 μM) of DOX and THP-ADM (Laboratoires Roger Bellon, Paris, France) were prepared in Dulbecco's PBS (pH 7.4, ionic strength $I = 0.152\,M$, with 1 mM EDTA). Drug concentrations in PBS solution were determined by their absorbances at 480 nm. Calf thymus DNA (Sigma Chemical Company, type I) was dissolved in Dulbecco's PBS. Concentration of DNA (phosphate) was estimated on the basis of a molar absorption coefficient $\varepsilon = 6\,600\,M^{-1}\,\mathrm{cm}^{-1}$ at 260 nm.

Abbreviations: DNA, deoxyribonucleic acid; DOX, doxorubicin; SERS, surface-enhanced Raman spectroscopy; THP-ADM, 4′O-tetrahydropyranyl adriamycin; PBS, phosphate buffered saline

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Cells

K562 is a human erythroleukaemia cell line, established from a patient with chronic myelogenous leukaemia in blast transformation (Lozzio and Lozzio 1975). K562 cells were kept in exponential growth at $5-8\times10^5$ in RPMI medium culture (Gibco) supplemented with 10% fetal calf serum (Serumed) and 2 m*M* L-glutamine. Cell growth and viability were determined by phase contrast microscopy.

SERS spectroscopy

SERS spectra were obtained on a DILOR Omars-89 Raman spectrometer connected to an Olympus BH-2 microscope equipped with a 100 × water immersion objective (Leitz fluotar), using the 514.5 nm line of an argon laser (Spectra-Physics, Model 2020-03). The laser power on the sample was 10 mW for the measurements in the macrochamber and less than 0.2 mW for the measurements under the microscope.

Silver hydrosol was prepared by reduction of silver nitrate with trisodium citrate as described (Nabiev et al. 1990a) and pre-aggregated by the addition of sodium perchlorate up to a final concentration of 0.05 *M*.

Stock solutions of antitumor drugs were dissolved in PBS at a concentration of 1 μM and introduced into the pre-aggregated silver hydrosol at a final concentration of 10^{-8}–10^{-10} *M*.

The population of treated cells (2×10^6 cells) was washed twice with PBS by centrifugation (1 000 rpm at 4 °C) and resuspended in 200 µl PBS. 100 µl of the cell suspension (10^6 cells) was introduced into 400 µl of silver hydrosol and after 30 min incubation the cells were precipitated, washed free of the hydrosol on the outside with PBS, and used for Raman measurements. The viability of cells incubated with silver hydrosol was checked by microscopy with 0.1% Trypan blue, the percentage of survival was always more than 95%.

Images of cells incubated in the presence or absence of the silver hydrosol were recorded on an image analyzer (SAMBA 2500, System for Analytical Microscopy in Biological Application, Alcatel TITN, Grenoble, France). For this, after the appropriate treatment, cells were washed twice, resuspended in PBS and observed under the microscope through a 100 × water immersion objective (Leitz Fluotar).

Results

SERS spectra of the antitumor drugs: DOX, THP-ADM and the complex of DOX with calf thymus DNA in vitro

SERS spectra of doxorubicin and THP-adriamycin adsorbed on silver hydrosol at a concentration of 10^{-8} *M* are shown in Fig. 1. At this concentration the best signal to noise ratio was obtained. Characteristic bands of these compounds could also be detected at concentrations as low as 10^{-10} *M* but contributions from impurities become important at such low concentrations. Increasing the drug concentration led to an increase in the fluorescence from the molecules in the bulk solution.

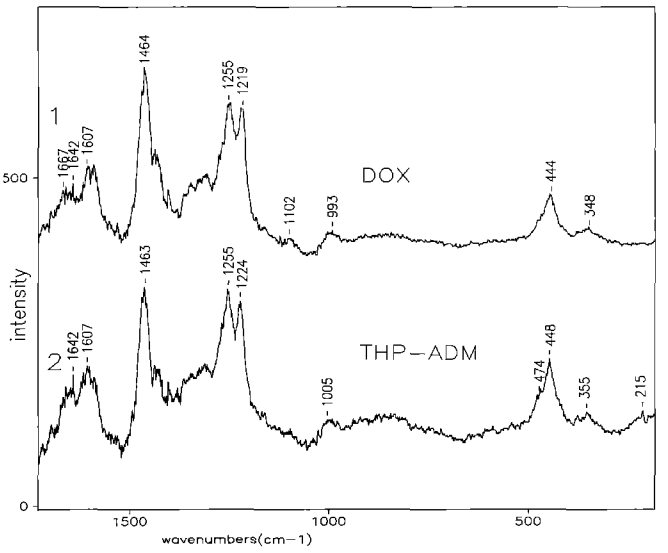

Fig. 1. SERS spectra of doxorubicin (DOX, 1) and THP-adriamycin (THP-ADM 2) adsorbed on silver hydrosol. Drug concentrations – 10^{-8} *M*

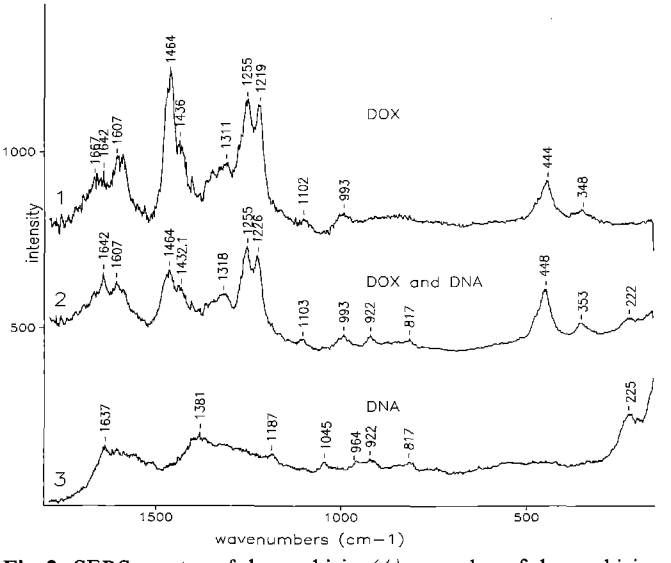

Fig. 2. SERS spectra of doxorubicin *(1)*, complex of doxorubicin with calf thymus DNA – 1 molecule of doxorubicin per 1 000 bp – *(2)*, and calf thymus DNA *(3)* adsorbed on silver hydrosol. Concentration of doxorubicin in free form as well as in a complex with DNA – 10^{-8} *M*

Figure 2 shows SERS spectra of DOX, of its complex with calf thymus DNA in vitro and of free calf thymus DNA adsorbed on silver hydrosol. The main differences associated with complex formation manifest themselves in changes of the relative intensities of the bands at 1 220/1 255 cm^{-1} (ν(C–O) + δ(C–O–H) + δ(C–H)) and 1 464 cm^{-1} (ν(C=C) + ν(C–C) + ν(C=O ··· H)) (Manfait et al. 1981) of the chromophoric part of the molecule as well as in an increase in intensity of the 1 642 cm^{-1} band assigned to ν(C=O ··· H) vibration. Calf thymus DNA did not give any significant contribution to the SERS spectrum of the complex under the experimental conditions used (Fig. 2).

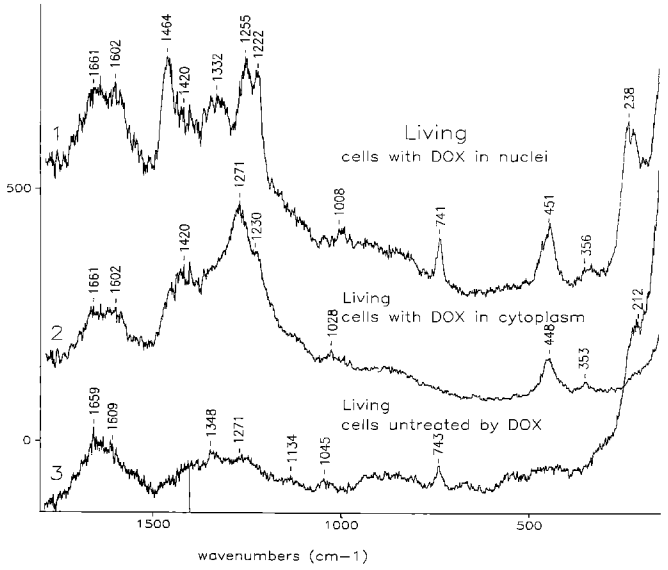

Fig. 3. SERS spectra of a population of living cells treated with doxorubicin and incubated with hydrosol *(1)*; untreated cells incubated with hydrosol containing doxorubicin *(2)* and untreated cells incubated with pure hydrosol *(3)*

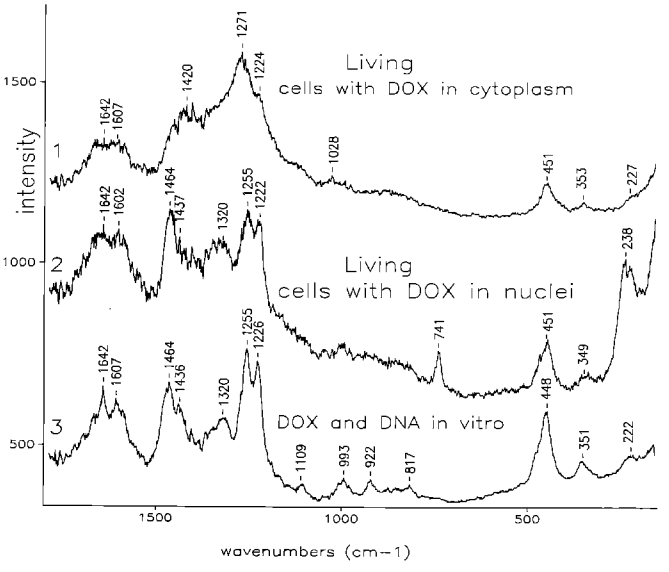

Fig. 4. SERS spectra of a population of untreated living cells incubated with hydrosol containing doxorubicin *(1)*; living cells treated by doxorubicin and incubated with hydrosol *(2)* and complex of doxorubicin with calf thymus DNA (1 mol of doxorubicin per 1000 bp) in vitro *(3)*

Population of living erythroleukaemic K562 cancer cell line

SERS spectra were detected from a population of living cancer cells incubated with silver hydrosol for 30 min (Fig. 3, curve 3). The striking feature of these spectra is the appearance of the 743 cm^{-1} band characteristic of the adenine ring breathing vibration, this is due to a number of destabilized regions of the nucleic DNA. However, in the DOX-DNA model the double-strand helix is rather stable, so a very small contribution of the destabilized regions does not allow the detection of this ring-breathing mode of adenine in the presence of hydrosol not activated

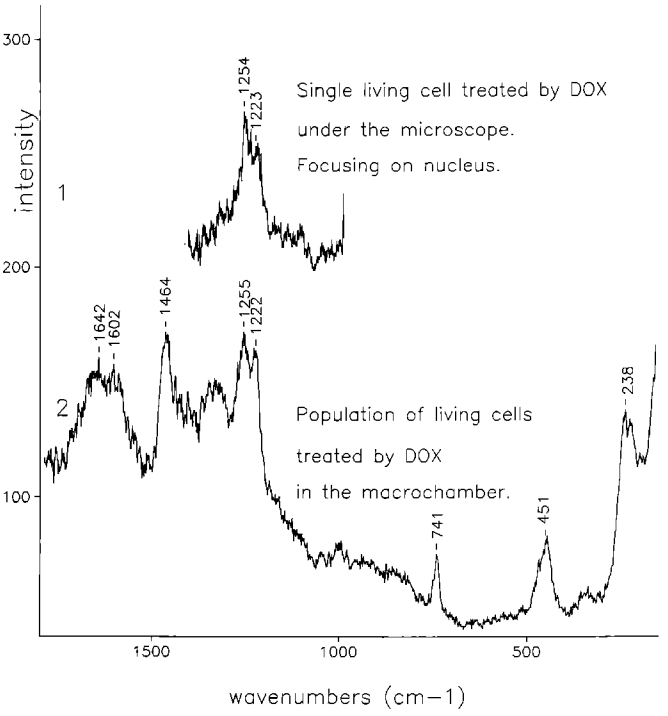

Fig. 5. SERS spectrum of the population of living cells treated with doxorubicin and incubated with hydrosol, and SERS spectrum obtained under the microscope by focussing a laser beam on the nucleus of a single living cell from this population

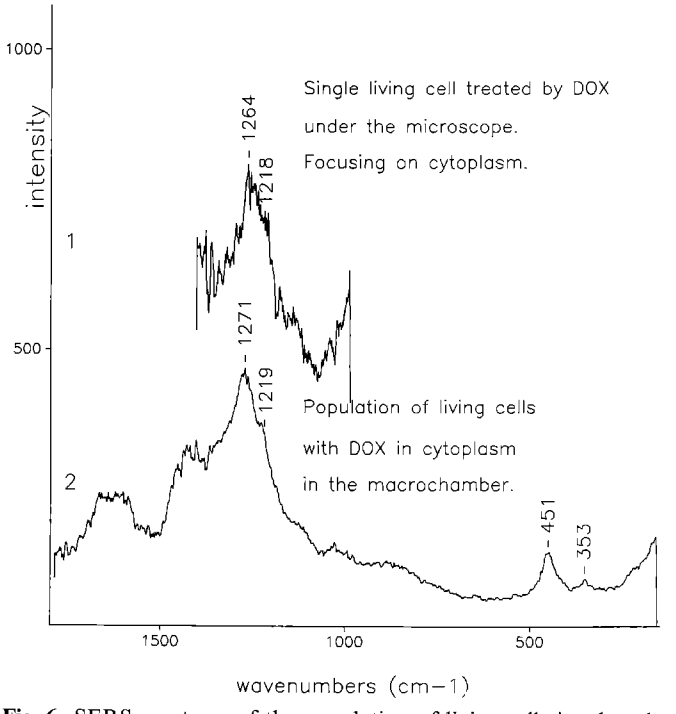

Fig. 6. SERS spectrum of the population of living cells incubated with hydrosol containing doxorubicin, and SERS spectrum obtained under the microscope by focussing a laser beam on the cytoplasm of a single living cell treated with doxorubicin and incubated with hydrosol

by Cl ions (Nabiev et al. 1990b). This marker band is not seen in the Raman spectrum of living cells which have not been incubated with silver hydrosol.

The SERS spectrum of living cancer cells treated with DOX for 1 h at 37 °C, washed with PBS, then incubated

with hydrosol for 30 min at room temperature (Fig. 3, curve 1), is very different from the spectrum of untreated cells incubated for 30 min in hydrosol containing DOX (Fig. 3, curve 2). It is very interesting to note that only in the first case do SERS signals from the adenine moiety appear in the spectrum (Fig. 3, curve 1).

The SERS spectrum of the cells treated with DOX corresponds closely to the spectrum of the in vitro complex of DOX with calf thymus DNA (Fig. 4, curves 2 and 3). The spectrum of cells untreated but incubated with the hydrosol containing this drug is very different from both of them as well as from the SERS spectrum of free DOX in the region 1200–1500 cm^{-1} (compare Fig. 4, curves 1–3 and Fig. 1, curve 2).

Single living erythroleukaemic cancer cells (line K562) under the microscope

The main representative regions of the interaction in the SERS spectra of a single living cancer cell treated with DOX obtained under the microscope with the laser beam focussing either on the nucleus (Fig. 5) or on the cytoplasm (Fig. 6) fully correspond to the SERS spectra of populations of the living cancer cells treated with DOX or incubated with the silver hydrosol containing this drug.

Discussion

Normal resonance Raman spectra of DOX and THP-adriamycin can be obtained at concentrations greater than 10^{-4} *M* and they have a very high contribution from the fluorescence background (Smulevich and Feis 1986; Manfait et al. 1981). The SERS spectra of these drugs (Fig. 1) are similar to their resonance Raman spectra reported earlier (Manfait et al. 1982) and correspond to the chromophoric part of the molecules. The resonance Raman spectrum of aclacinomycin A was obtained earlier but with a very high background due to the high quantum yield of fluorescence for this drug in the visible region (Nonaka et al. 1990). The SERS effect is accompanied by a strong quenching of fluorescence (Nabiev et al. 1990a; Smulevich and Feis 1986; Smulevich et al. 1988) and this provides the possibility of extending the range of molecules that can be investigated by this technique. Moreover, the ultrahigh sensitivity of SERS for this kind of molecule permits one to obtain spectra at concentrations down to 10^{-10} *M* and to develop a rapid method of detecting antitumor drugs in living cells.

The interaction of DOX with calf thymus DNA in vitro is accompanied by a strong decrease of the 1464 cm^{-1} band (Fig. 2) assigned to the $\nu(C=C)+\nu(C-C)+\nu(C=O\cdots H)$ vibration (Manfait et al. 1981). As was demonstrated (Sequaris et al. 1984), a penetration of Pt-coordinated compounds inside the double-stranded helix of DNA led to the disappearance of the vibrational bands of groups which are further than 5 Å from the metal surface. This effect indicates that the mechanism of SERS has a pronounced short-range character and this has been used extensively by us for the study of membrane protein topography (Abdulaev et al. 1987; Nabiev et al. 1990a). The decrease of the 1464 cm^{-1} band for the complex of DOX with DNA indicates the intercalation of a part of the chromophoric group of this drug within the double-stranded helix. The same result was obtained earlier by one of us (Manfait et al. 1982) using normal resonance Raman spectroscopy and demonstrated intercalation of DOX into the double-stranded helix in the GC-rich regions of DNA.

For the analysis of the interaction of doxorubicin with living cancer K562 cells we used three preparations:

(1) untreated cells were washed with PBS to remove culture medium, introduced into hydrosol and incubated there for 30 min at room temperature. The cells were washed with PBS to remove the hydrosol outside the cells before Raman measurements.

(2) cells were washed with PBS to remove culture medium, incubated with DOX for 1 h at 37 °C, washed with PBS to remove the DOX outside the cell and then introduced into the hydrosol in the same manner as for the first preparation.

(3) cells were washed with PBS to remove culture medium, introduced into hydrosol containing just 10^{-6} *M* DOX, incubated there for 30 min at room temperature, and washed with PBS to remove hydrosol as well as hydrosol-DOX complexes outside the cells.

The goal of procedure *(1)* was to introduce hydrosol micelles inside the living cells through endocytosis. We will call this preparation "untreated cells". Procedure *(2)* led to accumulation of DOX in the nuclei of the cells (Manfait et al. 1982) so we will call this preparation "living cells with DOX in the nuclei" or "living cells treated with DOX". Procedure (3) was used to incorporate DOX-hydrosol complexes into the cytoplasm through endocytosis and we will call this preparation "living cells with DOX in the cytoplasm".

Figure 3 shows that the signal of adenine appears in the SERS spectrum of untreated cells (curve 3). This means that the hydrosol was introduced into the living cells through endocytosis during the incubation of cells with the hydrosol. Besides, this has also been confirmed by image analysis (see Material and methods) showing also that the silver hydrosol is actually incorporated in the living cells (Fig. 7). The hydrosol used was more specific to adenine than to other nucleotides (Nabiev et al. 1985, 1990b) because it was activated by chloride ions contained in the PBS. The SERS spectrum of living cells with DOX in the nuclei is very different from the spectrum of cells with DOX in the cytoplasm (Fig. 3) but is closely correlated with the spectrum of the in vitro complex of DOX with calf thymus DNA (Fig. 4). The spectrum of cells with DOX in the cytoplasm is different from the spectrum of free DOX adsorbed on hydrosol (Fig. 1). This means that the DOX in the cytoplasm has some kind of target different from its target (DNA) in the cell nucleus.

Figures 3 and 4 show SERS spectra obtained from the population of cells in the macrochamber of the Raman spectrometer but we also succeeded in recording spectra from single cells under the microscope. The SERS spectra of single living cells treated with DOX following proce-

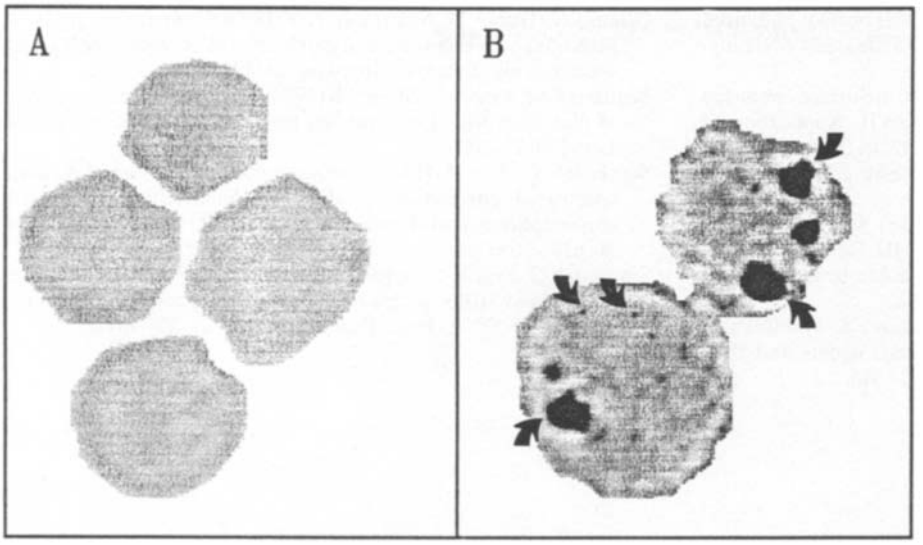

Fig. 7 A, B. Digitized images of K562 cells: **A** control; **B** incubated in the presence of silver hydrosol (see Materials and methods). In **B**, it can be seen that silver hydrosol is incorporated in the cells by endocytosis as compared to the control **A**

dure *(2)* and recorded by focussing a laser beam on the nucleus or on the cytoplasm are shown in Figs. 5 and 6, respectively. These spectra closely correspond to the spectra from the population of cells treated by DOX and of untreated cells incubated with hydrosol and DOX (i.e. cells with DOX in the cytoplasm). This confirms our suggestion about the existence of some intermediate target for DOX in the cytoplasm of living cells.

In this study we have demonstrated some differences in the mode of interaction of DOX between nucleus and cytoplasm. It has been believed that anthracyclines bind to DNA by intercalating between adjacent nucleotide base pairs, thereby inducing DNA strand damage and interfering with nucleic acid synthesis (Di Marco 1975; Nakamura et al. 1974). Recently another mode of action has been suggested for the cytotoxic effects of anthracyclines in which they produce free radical intermediates that react with molecular oxygen to form superoxide and other oxygen radicals that may contribute to DNA degradation (Berlin and Haseltin 1981; Kalyanaraman et al. 1980). For this reason, further investigations on the interaction of DOX and other anthracyclines with the nucleus and the cytoplasm, but performed on living cells, are considered important for the elucidation of biological effects of anthracyclines in cytotoxicity (Gigli et al. 1989) and differentiation (Millot et al. 1991). These studies could also help to understand the drug resistance mechanism which leads to different responses in clinical chemotherapy.

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References

Abdulaev NG, Nabiev IR, Efremov RG, Chumanov GD (1987) Retinal schiff base position relative to the surfaces of photoreceptor disk. FEBS Lett 213:113–118

Berlin V, Haseltin WA (1981) Reduction of adriamycin to a semiquinone-free radical by NADPH cytochrome P-450 reductase produces DNA cleavage in a reaction mediated by molecular oxygen. J Biol Chem 256:4747–4756

Chumanov GD, Efremov RG, Nabiev IR (1990) Surface-enhanced Raman spectroscopy of biomolecules. Part I. – Water-soluble proteins, dipeptides and amino acids. J Raman Spectrosc 21:43–49

Cotton TM (1988) The application of surface-enhanced Raman scattering to biochemical systems. In: Clark RJH, Hester RE (eds) Spectroscopy of surfaces. Wiley, Chichester, pp 91–153

Di Marco A (1975) Adriamycin (NSC 123127): mode and mechanism of action. Cancer Chemother 32:48–52

Gigli M, Rasoanaivo T, Millot J-M, Jeannesson P, Rizzo V, Jardillier J-C, Arcamone F, Manfait M (1989) Correlation between growth inhibition and intranuclear doxorubicin and 4′-iododoxorubicin quantitated in living K562 cells by microspectrofluorometry. Cancer Res 49:560–564

Kalyanaraman B, Prez-Reyes E, Mason RP (1980) Spin-trapping and direct electron spin resonance investigations of the redox metabolism of quinone anticancer drugs. Biochem Biophys Acta 630:119–130

Koglin E, Sequaris J-M (1986) Surface-enhanced Raman scattering of biomolecules. Top Current Chem 134:1–57

Lozzio CB, Lozzio BB (1975) Human chronic myelogenous leukemia cell-line with positive philadelphia chromosome. Blood 45:321–334

Manfait M, Bernard L, Theophanides T (1981) Resonance and preresonance Raman spectra of the antitumor drugs adriamycin and daunomycin. J Raman Spectrosc 11:68–74

Manfait M, Alix AJP, Jeannesson P, Jardillier J-C, Theophanides T (1982) Interaction of adriamycin with DNA as studied by resonance Raman spectroscopy. Nucl Acids Res 10:3803–3816

Millot J-M, Joly P, Benoist H, Manfait M (1991) Doxorubicin nuclear distribution necessary to induce erythroid differentiation of K562 cells. J Cell Pharmacol 3:1–8

Nabiev IR, Chumanov GD (1986) Surface-enhanced Raman spectroscopy of biopolymers: water-soluble proteins, amino acids and peptides, adsorbed by silver electrodes and hydrosols. Biofizika 31:183–190

Nabiev IR, Efremov RG (1983) Surface enhanced Raman spectroscopy of aromatic amino acids and proteins adsorbed by silver hydrosols. J Raman Spectrosc 14:375–379

Nabiev IR, Efremov RG, Chumanov GD (1985) The chromophore-binding site of bacteriorhodopsin. Resonance and surface-enhanced resonance Raman spectroscopy and quantum chemical study. J Biosci 8:363–374

Nabiev IR, Efremov RG, Chumanov GD (1988) Surface enhanced Raman spectroscopy and its applications to the study of biological molecules. Sov Phys Usp 31:241–262

Nabiev IR, Chumanov GD, Efremov RG (1990a) Surface-enhanced Raman spectroscopy of biomolecules. Part II. Application of short- and long-range components of SERS to the study of the structure and function of membrane proteins. J Raman Spectrosc 21:49–54

Nabiev IR, Sokolov KV, Voloshin ON (1990b) Surface-enhanced Raman spectroscopy of biomolecules. Part III. Determination of the local destabilization regions in the double helix. J Raman Spectrosc 21:333–336

Nakamura T, Tatsumi K, Higuchi T, Shirakawa S, Wakisaka G (1974) Mechanism of action of antileukemic agents and their clinical effects. Acta Haematol Jpn 37:302–315

Nonaka Y, Tsuboi M, Nakamoto K-J (1990) Comparative study of aclacinomycin versus adriamycin by means of resonance Raman spectroscopy. J Raman Spectrosc 21:133–141

Sequaris J-M, Koglin E, Malfoy B (1984) Inner and outer complexes of platinium with DNA probed by SERS spectroscopy. FEBS Lett 173:95–98

Smulevich G, Feis A (1986) Surface enhanced resonance Raman spectra of adriamycin, 11-deoxycarminomycin, their model chromophores and their complexes with DNA. J Phys Chem 90:6388–6392

Smulevich G, Feis A, Mantini AR, Marzocchi MP (1988) Resonance Raman and SERS spectra of antitumor anthracyclines and complexes with DNA. Ind J Pure Appl Phys 26:207–211