Intracellular molecular interactions of antitumor drug amsacrine (*m*-AMSA) as revealed by surface-enhanced Raman spectroscopy

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Abstract Cytotoxicity of several classes of antitumor DNA intercalators is thought to result from disturbance of DNA metabolism following trapping of the nuclear enzyme DNA topoisomerase II as a covalent complex on DNA. Here, molecular interactions of the potent antitumor drug amsacrine (*m*-AMSA), an inhibitor of topoisomerase II, within living K562 cancer cells have been studied using surface-enhanced Raman (SER) spectroscopy. The work is based on data of the previously performed model SER experiments dealing with amsacrine/DNA, drug/topoisomerase II and drug/DNA/topoisomerase II complexes in aqueous buffer solutions. The SER data indicated two kinds of amsacrine interactions in the model complexes with topoisomerase II alone or within ternary complex: non-specific (via the acridine moiety) and specific to the enzyme conformation (via the side chain of the drug). These two types of interactions have been both revealed by the micro-SER spectra of amsacrine within living K562 cancer cells. Our data suppose the specific interactions of amsacrine with topoisomerase II via the side chain of the drug (particular feature of the drug/topoisomerase II and ternary complexes) to be crucial for its inhibitory activity.

Key words: SER spectroscopy; Confocal microspectroscopy; Topisomerase II inhibitor; Amsacrine; Ternary cleavable complex; K562 cell line

1. Introduction

Several types of antitumor DNA intercalating agents, including anthraquinone (e.g., mitoxantrone, doxorubicine, etc.) and aminoacridine (e.g., amsacrine or *m*-AMSA) derivatives, have been shown to be able to trap the nuclear enzyme DNA topoisomerase II in the form of a cleavable complex on cellular DNA [1]. The mechanism by which cleavable complexes constitute biologically deleterious lesions is not clear but presumably involves some disruption of DNA metabolism [1]. In fact, DNA topoisomerase II [2] is the eucaryotic counterpart of bacterial gyrase and can alter the topology of DNA in a reaction involving the introduction of a transient double strand break into DNA, through which an intact helix can be passed [3]. This reaction involves the formation of a non-covalent enzyme/DNA complex which is in equilibrium with a covalent cleavable complex [4]. Topoisomerase II poisons such as *m*-AMSA stabilize the cleavable complex, preventing religation of the DNA strands [4,5]. The accumulation of the amsacrine-stabilised cleavable ternary complexes, sequestering strand breaks, has been shown to be an important factor in the cytotoxic action of the drug [6]. Nevertheless, the molecular mechanisms of the drug action against topoisomerase II are still to be defined.

Nowadays, using of intracellular molecular probes is recognized as one of the most effective approaches in diagnostics of pathology and assessment of cancer treatment efficiency. The anticancer drugs used for cancer cells treatment can be also considered as molecular probes. Optical microspectroscopic techniques, powerful and non-destructive, are particularly suitable to study molecular interactions between drugs and their pharmacological targets within living cancer cells [7]. At the molecular level, interaction of drugs with their potential cellular targets (DNA, enzymes) can be studied using the high molecular selectivity and sensitivity offered by SER technique. This kind of investigations has already enabled to propose models (consistent with corresponding data from crystallography, NMR, etc.) of the drug-target(s) complexes for the numerous inhibitors of topoisomerases I and II [8–13,16].

The non-perturbing character of the amsacrine adsorption on the silver colloid, demonstrated by comparative study of the drug by resonance Raman and SER techniques, allowed us to study the complexes of this drug with DNA, topoisomerase II and the ternary complexes [13]. Another advantage of the colloidal SER-active systems is their ability to penetrate through cellular membranes and, therefore, to play a role as an intracellular SER probe [14–16]. The present work is an extension of the studies with amsacrine to the cellular level by use of the confocal micro-SER technique. This novel method is realised by coupling of a Raman spectrometer with an optical microscope [14]. Confocal scheme of the signal filtration allows to achieve the spatial resolution of micron order and makes the approach very promising for the intracellular studies. Finally, the structural features of drug/target complexes in vitro and in living cells are compared.

2. Materials and methods

2.1. Chemicals

Stock solution of *m*-AMSA (amsacrine, Fig. 1) [17] at 10^{-2} M was supplied by Dr. J.-F. Riou (Rhône-Poulenc Rorer, France) and used as received. Further dilution in buffer (0.02 M Tris-acetate, pH 7.3, 2 mM NaCl, 5 mM $MgSO_4$) was made just before the experiments. The use of a low ionic strength (< 0.02) buffer allowed us to avoid the marked depressing of the association constant for amsacrine binding to DNA, observed by Wilson et al. [18] at elevated ionic strength (0.1). The buffer for the model complexes preparation contained 1 mM of ATP (Sigma), because it was shown to stimulate the cleavage reaction several fold [1].

Aqueous silver colloid was prepared by reducing silver nitrate with trisodium citrate as described [19] and pre-aggregated by the addition of a buffer containing magnesium sulphate (final concentration of $MgSO_4$ was 2.5 mM).

pBR322 plasmid was purchased from Boehringer Mannheim. Topoisomerase II was purified by J.F. Riou (Rhône-Poulenc Rorer,

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Abbreviations: Amsacrine (*m*-AMSA), 4′-(9-acridinyl-amino)-methanesulphon-*m*-anisidide); *o*-AMSA, 4′-(9-acridinyl-amino)-methanesulphon-*o*-anisidide; SER, surface-enhanced Raman scattering

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France) from calf thymus according to previously published procedures [20,21]. Topoisomerase was aliquoted and stored at −70°C, without detectable loss of activity, in a conservation buffer containing 10 mM Tris·HCl, pH 7.5, 20 mM 2-mercaptoethanol, 0.5 mM EDTA, 100 mg/ml bovine serum albumin, and 50% glycerol. One unit of topoisomerase II corresponds to the amount of enzyme necessary to relax (or decatenate) 50% of 0.5 mg of pBR322 DNA, when incubated for 30 min at 37°C under the assay conditions.

Preparation of the complexes for SER model measurements was as described before [13]. Briefly, the drug solution in the buffer was mixed with that of pBR322 and/or topoisomerase II, to obtain final molecular ratios: 1 drug per 15 base pairs of DNA and 50 U of topoisomerase II per 0.5 μg of DNA.

2.2. Cells

The K562 human erythroleukemia cell line was established from a patient with chronic myelogenous leukaemia [22]. Cells (10^6) were incubated in presence of 10 μM *m*-AMSA for 1 h at 37°C and washed twice with buffer by centrifugation (200×*g* at 4°C). Treated cells were then incubated with pre-aggregated silver colloid, centrifuged (5 min, 200×*g* at 4°C) for a better penetration of the colloid aggregates, and washed with buffer to eliminate non-penetrated aggregates. Very short spectra recording time (about 1 min) and very low, about 20 μW, laser power at the sample both permitted to carry out the measurements without damage of the cells by laser radiation.

2.3. Instrumentation

The 514.5 nm line of a Spectra-Physics, model 2020-03, argon laser was used as excitation radiation in both SER (10 mW at the sample) and micro-SER (20 μW under ×100 objective of microscope) measurements. No alteration of the spectra has been observed during the measurements. The SER spectra for the model experiments in solutions [13] were recorded with an OMARS-89 Raman Spectrometer (DILOR). The micro-SER spectra for the intracellular studies were recorded with a confocal Raman micro-spectrometer LABRAM (DILOR) with a spatial resolution of 1 μm [14]. The microscope of the micro-spectrometer was equipped with a CCD camera to obtain TV images of the cells. The images and spectra were recorded with a 'LABRAM' software package.

Presented spectra of the model experiments are the result of at least three independent measurements. The resulting intracellular SER spectrum shown is the average of more than 10 spectra recorded from the points with similar localisation relative to the main cellular compartments.

3. Results and discussion

Previous to the intracellular investigations, a detailed study of the acridine chromophore has been performed in vitro by resonance Raman and SER techniques [13]. The resonance Raman and SER spectra of amsacrine were very similar, when excited at the same wavelength [13]. Thus, under conditions used, the adsorption on the surface of the silver colloids does not perturb the acridine chromophore. SER spectral patterns of amsacrine have been analysed by use of the structurally close acridine derivatives, *o*-AMSA and 9-aminoacridine (Fig. 1A). As can be seen in the Fig. 1A, lowering of the symmetry of the acridine chromophore, caused by the appearance of the side chain (anilino rings of *o*- and *m*-AMSA), affects numerous Raman bands in the spectral region between 1605 and 1400 cm^{-1}. However, the bands of amsacrine at 1520, 1437, 1413, 1260 and 1237 cm^{-1} are sensitive not only to the presence of the anilino ring but also to the position of its substituents (see spectra 1 and 2 of meta- and ortho-isomers, respectively).

These findings have been employed to interpret the SER data for the model complexes of the drug with plasmid DNA, topoisomerase II, and the topoisomerase II-mediated ternary cleavable complexes, prepared in aqueous buffer solutions (Fig. 1B). The main conclusions concerning these complexes can be summarised as follows [13]:

(1) SER spectra gave indications of the intercalation of the planar acridine moiety of *m*-AMSA within DNA. The inter-

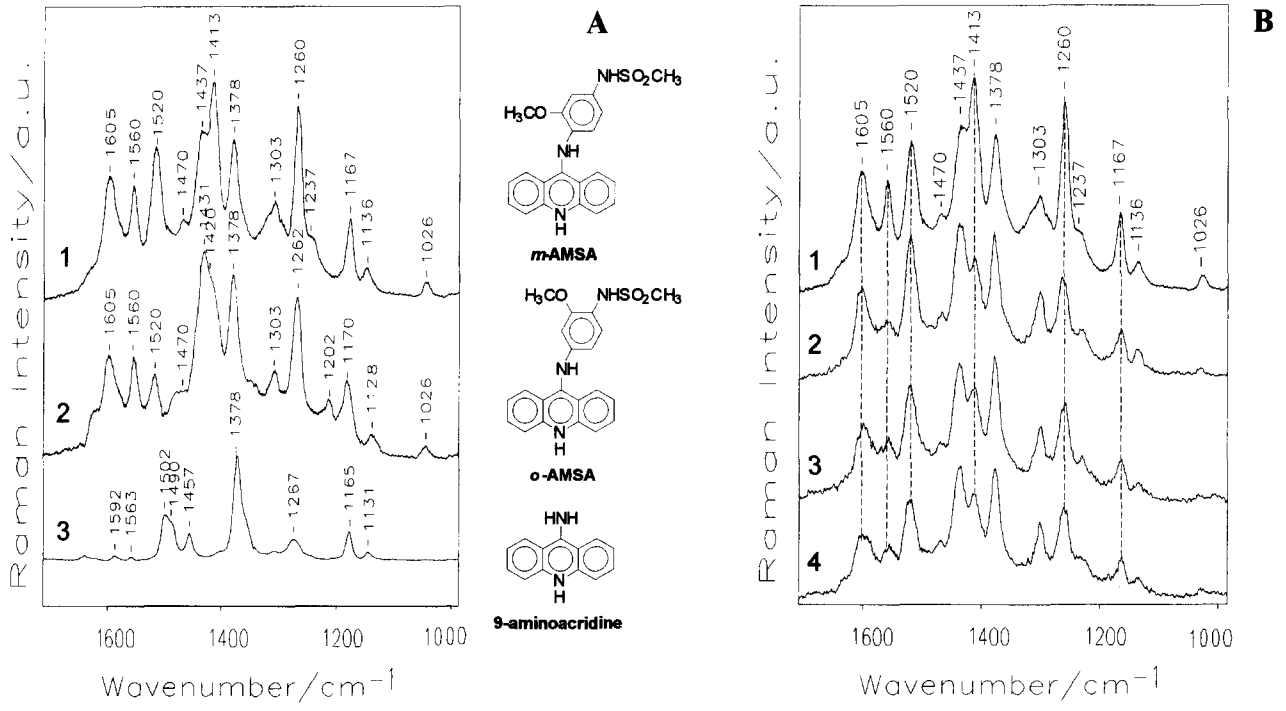

Fig. 1. A: Structural formulae and SER spectra of amsacrine (*m*-AMSA) (1), *o*-AMSA (2), and 9-aminoacridine (3) at 5×10^{-7} M in Tris-acetate aqueous buffer, pH 7.3. B: SER spectra of free amsacrine (1) and in the model amsacrine/DNA (2), amsacrine/topoisomerase II (3), and amsacrine/DNA/topoisomerase II (4) complexes.

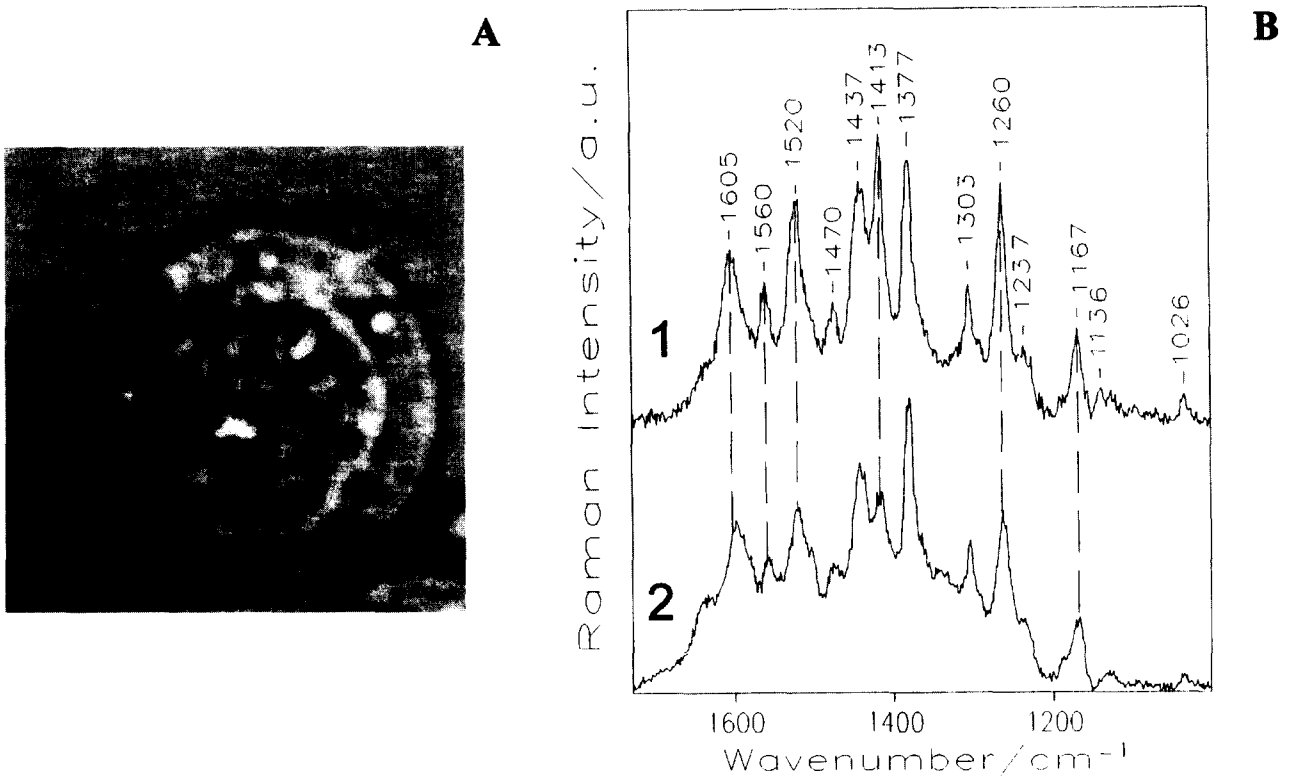

Fig. 2. A: TV image of the K562 cancer cell treated with amsacrine and containing silver colloidal particles (indicated with arrows). B: Micro-SER spectra of free amsacrine (1) and from colloid particles situated in the vicinity of the nuclea of living K562 cancer cells (2).

calation involves a π–π interaction between the drug and DNA base pairs. No contacts via the nitrogen of the acridine ring can be assumed, since the spectral changes for the drug/DNA complexes were different from the spectral features related to acridine deprotonation (downshift of the amsacrine band at 1260 cm^{-1} and the intensity increase of the 1560 cm^{-1} band, data not shown). Arrangement of the external anilino ring in the minor groove of DNA is supposed to be stabilised by interactions between the NH group of the side chain of the drug and negatively charged phosphates or edges of the DNA.

(2) SER data indicated the ability of amsacrine to interact via its side chain with the enzyme alone. These interactions, specific to enzyme conformation, were not observed for the ortho-isomer of the drug. Thus, it has been shown that the side chain recognition as well as the spatial position and/or relative orientation of the acridine and anilino moieties of amsacrine are crucial to interact with the reaction sites of the enzyme.

(3) The data of the SER experiments in solution suppose that in the ternary complexes of amsacrine both DNA intercalation and drug/topoisomerase II interactions take place. The suggested model for the ternary complex of the drug is as follows: the acridine moiety serves as the DNA binding anchor, whereas the side chain of the drug, occupying the DNA groove, interacts with topoisomerase II.

The next stage of this study was an attempt to validate the in vitro modelling by intracellular data. In order to analyse intracellular micro-SER spectral patterns, the micro-SER spectra of free amsacrine (spectrum 1 in Fig. 2B) were previously recorded with a confocal Raman micro-spectrometer (see Section 2). These spectra have been obtained from isolated colloidal aggregates, incubated with the drug solution in buffer, in the absence of cells. The micro-SER spectra were similar to the SER spectra, except for a slight difference in the relative intensities of the Raman bands. This is due to the different detection systems used for the SER and micro-SER measurements (see Section 2).

Then micro-SER measurements were performed on living K562 human erythroleukemia cells. These were treated with amsacrine and incubated with silver colloids as described in the Section 2. Visual analysis of the cells under microscope indicated a moderate penetration of colloid aggregates into the cells. The viability of cells incubated with silver colloids was checked by phase contrast microscopy and using 0.1% Trypan blue. The percentage of survival was more than 95% (Fig. 2A).

On going to the colloidal aggregates within the cells, the micro-SER spectra of amsacrine were modified (Fig. 2B). Thus, we cannot assume presence of the free drug within the cells. It should be noted that the SER signal of amsacrine from small colloidal micelles located in the cytoplasm, distant from the nucleus, was negligible. Better micro-SER spectra of amsacrine were recorded from the silver aggregates, situated in the vicinity of the nucleus of the living K562 cells. All these spectra were recorded in the same conditions: laser beam was focused onto the nucleus and on a silver aggregate. A large number of cells was analysed. From cell to cell, the spectral features observed for a given localisation, with respect to the cellular compartments, were well reproducible. This allowed us to analyse the spectra in order to determine the general spectral pattern for a given cellular compartment.

As can be noted in the Fig. 2B, the signal-to-noise ratio and spectral resolution of the micro-SER spectra in the vicinity of the nucleus were very good. The decrease of the relative in-

tensity of the bands at 1605, 1560, 1520, 1413, 1260 cm^{-1} was clearly pronounced. It is very important to underline that these spectral modifications (Fig. 2B) were very similar to those in the SER spectra of amsacrine in the model complexes in the presence of topoisomerase II (Fig. 1B).

As was described before based on the data of the SER experiments [13], two kinds of amsacrine/target interactions in the model complexes are assumed: (1) interactions via the acridine moiety, indicated by loss of the intensity of the bands at 1560, 1413 and 1260 cm^{-1}. These have been observed for amsacrine and for its ortho-isomer in the complexes with DNA and/or topoisomerase II (Fig. 1B). The interactions of this kind are not specific to the enzymatic conformation, since they have been observed for the drug in the presence of deactivated topoisomerase II [13] (data not shown). (2) interactions via the side chain of the drug, indicated by decrease of the bands at 1604 and 1520 cm^{-1}. These have been observed for amsacrine (and not for *o*-AMSA) in the presence of active topoisomerase II (alone or within ternary complexes, spectra 3 and 4 in Fig. 2, respectively). These interactions are specific to the enzyme conformation, since they disappeared when the complexes were prepared with deactivated topoisomerase II [13].

The observation of both (1) and (2) spectral features in the intracellular micro-SER spectra (Fig. 2B) indicates both specific and non-specific molecular interactions of amsacrine within living K562 cancer cells. Although these two types of interactions are both involved in the molecular mechanisms of action of amsacrine, however, specific ones should play a crucial role in the inhibitory activity of the drug, since they form a particular feature of the drug/topoisomerase II and ternary complexes and are not observed for the inactive ortho-isomer of the drug.

It is noteworthy that the only feature differing the SER spectra of amsacrine within drug/DNA/topoisomerase II complexes (spectrum 4 in Fig. 1B), from those of drug/topoisomerase II (spectrum 3 in Fig. 1B), was a less pronounced decrease of the intensity ratio of the 1605/1595 cm^{-1} bands in the ternary complexes. From this point of view, the intracellular micro-SER spectra are closer to those of the drug/topoisomerase II complexes. However, the described feature is too slight to be indicative of the preference for the amsacrine/topoisomerase II complexes rather than ternary ones as corresponding to the intracellular data. In fact, although the biological activity of amsacrine is supposed to be realised by stabilisation of cleavable ternary complexes [1,5], our data do not exclude that the drug/topoisomerase II direct interactions could be responsible for inhibition of this nuclear enzyme.

For this reason, further investigations of the interaction of *m*-AMSA and other DNA intercalating and non-intercalating topoisomerases poisons with the components of the cellular nucleus and the cytoplasm, performed on living cells, are considered important for the elucidation of biological effects of these drugs in cytotoxicity, differentiation and programmed cell death or apoptosis. These studies could also help to understand the drug resistance mechanisms which lead to different responses in clinical chemotherapy.

Our data demonstrate great capabilities of the micro-SER technique as a non-destructive intracellular probe and support the model experiments as a good assumption of the intracellular molecular interactions.

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