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Studies on the interaction of adriamycin with d-$(TGATCA)_2$ by proton nuclear magnetic resonance spectroscopy, time-resolved fluorescence measurement, diffusion ordered spectroscopy followed by structural refinement using restrained molecular dynamics approach

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ABSTRACT

Adriamycin is one of the most potent anticancer anthracycline drug having aromatic chromophore and an amino sugar moiety. We report here the solution structure of 2:1 adriamycin–d-$(TGATCA)_2$ complex which has been determined using restrained molecular dynamics. Sequential NOE (nuclear Overhauser effect) connectivities between T1pG2 and C5pA6 steps are not observed on the intercalation of the drug chromophore at these base pair steps. Presence of several other intermolecular NOEs, that is, T1CH_3—7H, T1CH_3—10eqH and C5H6—4OCH_3 corroborate the same. The specificity of interaction arises from the *O*-glycosidic dihedral bond C7—O7—C1′—C2′ (133°), positioning of NH_3^+ moiety in minor groove, conformation of ring A and daunosamine sugar. Besides this, diffusion ordered spectroscopy (DOSY) studies prove the formation of complex and time-resolved fluorescence measurement studies provide evidence for shortening of decay time on complex formation. The nonspecific interactions as well as those essential for molecular basis of drug action are discussed along with the specificity of interactions in the drug–DNA complex, which is responsible for the anticancer action of the drug.

1. Introduction

Anthracycline antibiotics are of particular interest as potential anti tumor agents, which bind to both DNA and the DNA binding protein topoisomerase-II [1]. The biological activities of different anthracycline based drugs depend on the minor modifications in their chemical structure and accordingly several analogues have been developed. The daunomycin family of anthracycline antibiotics has been used effectively in treating a variety of cancers [1–4]. Adriamycin has the widest spectrum of antitumor activity and is used for solid tumors. It differs (Fig. 1a) from the parent drug daunomycin in replacement of one hydrogen atom of methyl group at carbon 14 position (9COCH_3 moiety) by a hydroxyl group (9CO-CH_2OH). There has been a constant search to look for new ligands or modify the existing drugs in order to overcome the adverse effects of cell toxicity or tumor-resistance. For structure-based design of new ligands the knowledge of exact conformational behavior is necessary. A study of DNase I digestion patterns have identified 5′TG as the preferred binding site [5,6] besides 5′CG which was observed as the most frequent dinucleotide in all protected regions [7]. Subsequently high resolution DNase I foot printing titration experiments [6] identified triplets of base sequences, indicating that either A or T may occupy the position neighboring the intercalation site.

X-ray crystal structure analysis of daunomycin [8] and 4′-epiadriamycin [9,10] with d-$(TGATCA)_2$ and d-$(TGTACA)_2$ hexamer sequences have shown that the complexes are differently stabilized through O4, O5, O13 and NH_3^+ groups of the drug in these complexes. It has been suggested [11] that glycosidic bond C7—O7—C1′—C2′ may adopt different conformations, which will result in flexibility in positioning of NH_3^+ group in minor groove of DNA close to the intercalation site. Also BII conformation of DNA may be stabilized due to intercalation. We have set out to examine the solution structure and dynamics of these complexes using nuclear magnetic resonance techniques combined with molecular modeling and other biophysical technique. We present here our results on interaction of adriamycin with d-$(TGATCA)_2$ (Fig. 1b) using NMR which provides information about the system in physiological condition and also yield insight into dynamic processes. The existence of bound and free resonances of imino protons of DNA clearly demonstrates that the drug indeed binds to

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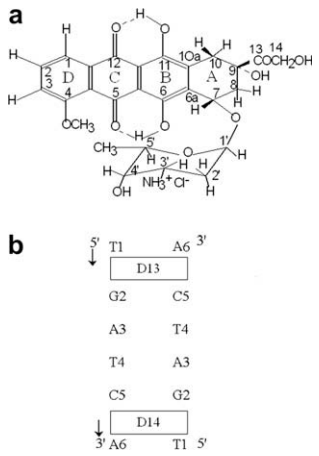

Fig. 1. (a) Molecular structure of adriamycin (b) Schematic representation of the 2:1 adriamycin–d-$(TGATCA)_2$ complex.

the DNA hexamer and there is a slow exchange of free and bound DNA on NMR time scale at low temperature. This palindromic sequence is chosen as firstly, it is the oncogene sequence and secondly, 5′TG is the preferred binding site followed by either A or T adjacent to the intercalation site for the interaction of the drug to the DNA sequence [6,7]. We have obtained conformational parameters, correlations between them and intermolecular contacts to establish the mode of binding. Along with them, DOSY and time-resolved fluorescence measurement studies are also done to get evidence of complex formation.

2. Experimental

The deoxyribonucleic acid sequence d-$(TGATCA)_2$ was purchased from Microsynth, Switzerland. Deuterium Oxide (D_2O), with isotopic purity 99.96% and adriamycin were purchased from Sigma–Aldrich Chemicals Pvt. Ltd. USA. Sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS), an internal NMR reference was purchased from Merck Sharp and Dohme Canada Ltd., Canada. All other chemicals like Na_2HPO_4 and NaH_2PO_4 and ethylene diamine tetra acetic acid (EDTA) are of analytical grade and purchased from E. Merck, India Ltd. Solution of adriamycin (20.49 mM) was prepared by dissolving a known quantity of sample in 90% water and 10% D_2O. The final concentration is checked by absorbance measurements at wavelength of 480 nm using Cary Bio 100 Spectrophotometer. The extinction coefficient (ε value used for adriamycin is $\varepsilon = 11{,}500\ M^{-1}\ cm^{-1}$. Solution of deoxyoligonucleotide, d-$(TGATCA)_2$ (3.42 mM, duplex concentration) was prepared by dissolving a known quantity of sample in deuterated phosphate buffer (20.0 mM) of pH = 7.0 having 50 mM Na salt. The sample of d-$(TGATCA)_2$ was dissolved in 540 μl of deuterated phosphate buffer and 60 μl of D_2O and its concentration was determined by absorbance measurements at 260 nm. Using extinction coefficient ($\varepsilon = 61{,}400\ M^{-1}\ cm^{-1}$). Ethylene diamine tetra acetic acid (EDTA), 0.1 mM, was added to suppress any paramagnetic impurity, which may cause line broadening during NMR measurements. Typically 1 μl of 0.1 M solution of DSS was added to the complex of d-$(TGATCA)_2$ and adriamycin as an internal reference. 3.42 mM d-$(TGATCA)_2$ and 20.49 mM adriamycin samples were taken as a stock solution for preparation of complex. A complex of d-$(TGATCA)_2$ and adriamycin was prepared by titration. 200 μl of 20.49 mM adriamycin was added in steps of 10 μl to 0.6 ml of 3.42 mM d-$(TGATCA)_2$ sample during titration in order to make the complex of adriamycin: d-$(TGATCA)_2$ D/N = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.8 and 2.0. All proton NMR experiments were carried out at Central NMR Facility, Indian Institute of Technology Roorkee and were recorded on Bruker Avance 500 FT-NMR spectrometer equipped with computer having Topspin (1.3 version). Typical parameters for one-dimensional NMR experiments are: pulse width = 10–12.5 μs (30° pulse); no. of data points = 128–256 K; spectral width = 5000 Hz; no. of scans = 64–128 and digital resolution = 0.25–0.5 Hz/point. Receiver gain was optimized in each instance to obtain the best signal to noise ratio. In temperature variable experiments, constant temperature is maintained in the range 275–328 K using temperature control accessory. 2D phase-sensitive NOESY experiment on d-$(TGATCA)_2$ and its complex with adriamycin were carried out at 275 K in 90% H_2O and 10% D_2O with variable mixing times (τ_m) 200 and 300 ms. Typical parameters for 2D experiments were: 1–2 K data points along t_2 dimension; 512 free induction decays in t_1 dimension; pulse width ≈ 9.5–12 μs; spectral width ≈ 5000 Hz; no. of scans = 64–128; digital resolution 2.30–4.60 Hz/point and relaxation delay ≈ 2.0 s. 2D NOESY spectra were recorded at two mixing times, τ_m = 200 and 300 ms. The intensities of cross peaks of 2D NOESY spectra at τ_m = 200 ms were used to extract the experimental restraints of inter-proton distances as the spin diffusion effect was negligible at this mixing time, therefore not considered during inter-proton distance calculations. CH5—CH6 peak of cytosine was used as the reference using a distance = 2.45 Å.

2.1. Time-resolved fluorescence measurements

Time resolved fluorescence decays were obtained by the time-correlated single-photon counting method on the spectrofluorometer (model FluoroLog-TCSPC, make HORIBA Jobin Yvon Spex), used for the lifetime measurement study. The excitation source (λ_{ex} = 470 nm) was a fixed-wavelength NanoLED. The emission was detected at the emission wavelength (λ_{em} = 593 nm). The fluorescence emission of the drug and its complex with d-$(TGATCA)_2$ was counted by a micro channel plate photo multiplier tube, after passing through the monochromator and processed through constant fraction discriminator (CFD), time-to-amplitude converter (TAC) and multi channel analyzer (MCA). All measurements were performed at 298 K in water. The fluorescence decay was obtained and further analyzed by using the software, DAS, provided by FluoroLog-TCSPC instrument.

2.2. Diffusion ordered spectroscopy (DOSY)

The DOSY experiment is the measure of diffusion coefficients by NMR. The relation between translational self-diffusion and the measurable NMR parameters [12] is:

$$A/A_0 = -\exp\left[D_{t\lambda H^2}\delta^2 G_z^2(\Delta - \delta/3)\right] \quad (1)$$

where A is the measured peak intensity (or volume), A_0 is the maximum peak intensity, D_t is the translational diffusion constant (in cm^2/s), γH is the gyromagnetic ratio of a proton ($2.675197 \times 10^4\ G^{-1}\ s^{-1}$), δ is the duration of the gradient, δ is the time between gradients and G_z is the strength of the gradient (in G/cm). Data can be plotted as $-\ln(A/A_0)$ versus $\gamma H^2\delta^2 G_Z^2$ ($\Delta - \delta/3$). The slope of the line gives the value of D_t. The pulse program used is pulsed gradient spin echo (stimulated echo sequence incorporating bipolar gradients) sequence modified with binomial water suppression. The gradient strengths were incremented as a square dependence in the range from 1 to 32 G cm^{-1}. The diffusion time (Δ) and the duration of the magnetic field gradients (δ) were 100 and 6 ms, respectively. Other parameters include a sweep width of 6000 Hz, 32 K data points, 1024 transients and an acquisition time of 2.7 s and relaxation delay of 2.0 s. It has been developed in order to facilitate the complex mixture analysis without physical