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Hetero-association of anticancer antibiotics in aqueous solution: NMR and molecular mechanics analysis

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Abstract

In order to investigate the effect on combinations of aromatic antibiotics used in chemotherapy, the hetero-association of the antitumour antibiotics actinomycin D (AMD) with daunomycin (DAU) or novatrone (NOV) has been studied by the methods of 1D- and 2D 500 MHz ^{1}H-NMR spectroscopy and molecular mechanics calculations. The experimental concentration and temperature dependences of the proton chemical shifts of mixtures of the aromatic drugs have been analyzed in terms of a modified statistical–thermodynamical model of hetero-association to give the equilibrium reaction constants, the thermodynamical parameters (ΔH, ΔS) of hetero-association of AMD with DAU or NOV and the limiting values of proton chemical shifts of the molecules in the hetero-complexes. The most favorable averaged structures of the 1:1 DAU–AMD and NOV-AMD hetero-association complexes have been determined using both the limiting values of proton chemical shifts of the molecules and molecular mechanics methods (X-PLOR software). The results show that intermolecular complexes between DAU–AMD and NOV–AMD are mainly stabilized by stacking interactions of the aromatic chromophores, although the DAU–AMD hetero-complex has additional stabilization, which may be explained by an intermolecular hydrogen bond between a carbonyl group of ring C of DAU and the NH group of D–Val of the pentapeptide side chain ring of AMD. The relative content of each type of molecular complex in the mixed solution has been calculated at different values of the ratio (r) of the initial concentrations of DAU and AMD. It is found that the contributions of hetero-complexes to the general equilibrium in solution are predominant at quite different values of r, *viz*. at $r>12$ for AMD with NOV and at $r>2$ for AMD with DAU, compared to $r>0.3$ for the DAU–NOV system observed previously. It is concluded that anticancer drugs have quite different affinities for formation of hetero-complexes with other aromatic antibiotics in aqueous solution, which may need to be taken into consideration for their use in combination chemotherapy.

Keywords: Hetero-association; Anticancer drugs; Daunomycin; Actinomycin D; Novatrone; NMR spectroscopy

1. Introduction

Combination chemotherapy, i.e. when several drugs are administered either simultaneously or sequentially, has proved to be very effective in the treatment of many human diseases, in particular malignant tumours [1–3]. Aromatic antitumour antibiotics, such as doxorubicin (DOX), daunomycin (DAU), mitoxantrone (also named novatrone, NOV), actinomycin D (AMD), amsacrine (AMSA), are widely used in clinical practice as basic components of combinations of drugs designed for treatment of various types of human cancers. For example, the combination of DOX+AMD is highly effective against various types of sarcomas [4,5], DOX+NOV is mainly used against breast cancer [6], and DOX+AMSA and NOV+AMSA are effective against leukaemia [7,8]. It should be noted that the concentrations of anticancer drugs used in chemotherapy are, as a rule,

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relatively small in blood plasma [1] so that one might not expect substantial interactions between drug molecules in vivo. However, it is significant that stock concentrations, C_0, (or doses of antibiotics to be administered) depend on chemotherapeutic regimen used but are typically in the millimolar range [1]. For example, for the antibiotic AMD C_0 ca. 0.4 mM, for the anticancer drugs NOV and DAU C_0 is 1–4 mM, and for other antibiotics concentrations of stock solutions may be 1–2 orders higher [1]. It has been shown that at millimolar concentrations in aqueous solution, there is a strong tendency for aromatic anticancer drugs to exhibit self-association forming dimers (AMD) and higher order aggregates (DOX, DAU, NOV), which are stabilized by stacking interactions of the aromatic chromophores [9–12]. It has been also shown that different types of biologically-active aromatic molecules form stacked hetero-associated complexes in aqueous solution, e.g. the above-mentioned anticancer antibiotics may complex with caffeine [13,14], riboflavine [15], chlorophylline [16] and aromatic dyes [17,18]. Hence it is likely that self- and hetero-association reactions of aromatic anticancer drugs take place in the stock solutions used for combination chemotherapy, which may affect the pharmacokinetics of these drugs and consequently their medico-biological activity. Moreover, the rate of metabolic activation/deactivation processes of antibiotics in the hetero-complex may be different from the kinetics of transformation of free drug in a biological fluid. It has been proposed that such a phenomenon is likely to be the preferred mechanism to explain the rate of degradation of DOX and other aromatic carcinogens on binding with riboflavin [19,20]. In some cases the hetero-association of aromatic molecules may lead to formation of stable hetero-complexes in solution as they are energetically more favorable than self-association of the constituent drugs. Our previous investigations have shown that stacked complexes between the antibiotic DAU and the aromatic dyes, proflavine and ethidium bromide, are additionally stabilized by intermolecular hydrogen bonds in aqueous salt solution [17,18]. Hence it is likely that physico-chemical investigations of the distinctive features of the interactions between aromatic antibiotics under physiological conditions are very important for the development of chemotherapeutic regimes involving combinations of these drugs.

In this work 500 MHz ^{1}H NMR (1D and 2D) spectroscopy and molecular mechanics methods have been used to investigate the formation of hetero-complexes between the aromatic antitumour antibiotics actinomycin D (AMD) and either novatrone (NOV) or daunomycin (DAU) in aqueous salt solution. The structures of AMD, DAU and NOV are shown in Fig. 1. The thermodynamic parameters of complexation of NOV–AMD and DAU–AMD in aqueous salt solution have been determined from the experimental concentration and temperature dependences of proton NMR chemical shifts of the interacting molecules, and the results compared with those for similar studies on the hetero-

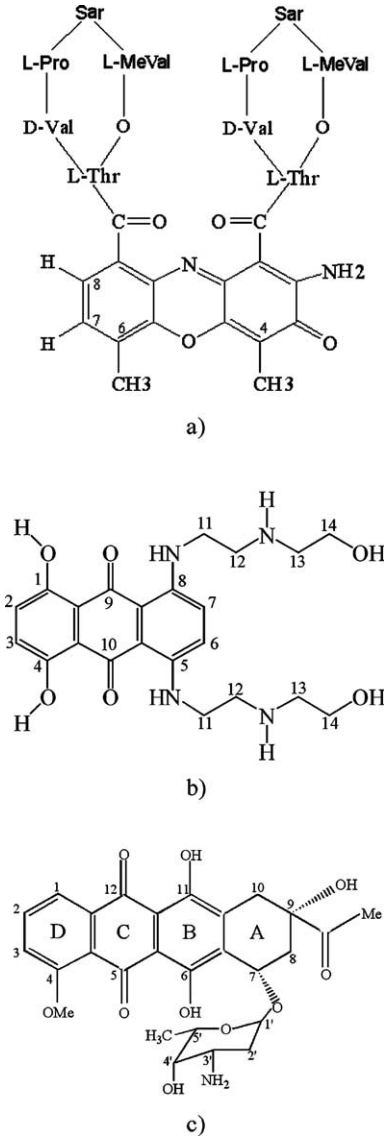

Fig. 1. Chemical structures of the anticancer antibiotics: a) actinomycin D (AMD); b) novatrone (NOV); c) daunomycin (DAU).

association of NOV and DAU under the same solution conditions [21]. Such investigations provide information on the nature of the physical forces involved in the complex-action of aromatic antitumour antibiotics and give some insight into the molecular basis of the pharmacological activity of drugs used in combination chemotherapy.

2. Materials and methods

2.1. NMR spectroscopy

The antitumour antibiotics (Fig. 1), actinomycin D (AMD) and novatrone (1,4-dehydroxy-5,8-bis [[2-(2-hydroxyethyl) amino] ethyl] amino]-9,10-antracenedione, NOV), were purchased from “Sigma” and daunomycin (DAU) from “Fluka” and were all used without further

purification. The samples were lyophilized from D_2O and re-dissolved in 0.1 M phosphate buffer in 99.95% D_2O, pD=7.1, containing 10^{-4} M EDTA. The concentrations of the stock solutions of the aromatic molecules were measured spectrophotometrically on appropriate dilution using the following molar absorption coefficients: for NOV, ε=7050 M^{-1} cm^{-1} (λ=645 nm) [22]; for AMD, ε=24 500 M^{-1} cm^{-1} (λ=440 nm) [23] and for DAU, ε=11500 M^{-1} cm^{-1} (λ=477 nm) [24,25]. 1D and 2D ^{1}H NMR spectra were recorded on a Bruker 500 MHz DRX spectrometer and the sample temperature was regulated using a Bruker BVT-3000 unit.

All NMR measurements were made in the fast-exchange condition on the NMR time-scale. Chemical shifts were measured relative to an internal reference TMA (tetramethylammonium bromide) and recalculated with respect to DSS (sodium 2.2 dimethyl 2-silapentane-5-sulphonate), i.e. $\delta_{DSS}=\delta_{TMA}+3.178$ (ppm). Signal assignments of the non-exchangeable protons of the drugs were obtained using two-dimensional homonuclear TOCSY, NOESY and ROESY experiments and the method of preparation of samples and performance of the NMR experiments have been described in detail elsewhere [10–12].

It should be noted that suitable conditions for NMR experimental investigations had to be found for the NOV–AMD system because the solubility of NOV increases with increasing temperature, whereas for AMD it decreases over the same temperature range. An optimum temperature of T=312 K was used so that NOV and AMD did not precipitate over the whole range of concentrations studied for measurements of chemical shifts. It was also found that AMD had limited solubility in the mixed solution with DAU and, therefore, chemical shifts measurements of the non-exchangeable protons of the aromatic molecules in the AMD–DAU system were made as a function of concentration of DAU in the range from 3.22 to 0.25 mM, whilst keeping the concentration of AMD constant ($C_{AMD}=P_0$=0.26 mM). The temperature dependences of proton chemical shifts for the AMD–DAU mixture were measured at constant concentrations of drug molecules in the temperature range 273–333 K.

2.2. Molecular mechanics calculations

Calculations of the most probable spatial structures of the 1:1 DAU–AMD and NOV–AMD complexes have been made by the methods of molecular mechanics using X-PLOR (version 3.851) [26] with the Charmm22 force field [27]. Modeling of the water–salt environment of the intercalated complexes was made with 1100 TIP3P water molecules [28] placed in a rectangular box (1100 molecules). The topology of the AMD, DAU and NOV molecules and parameterization of their atomic interactions were created by the XPLO2D program [29] using crystal structures from the PDB data bank [30]. Parameters of non-valent interactions corresponded to the MM3 force field [31].

3. Results

3.1. NMR measurements

The structural and thermodynamical parameters of the hetero-association between AMD and DAU or NOV were determined from chemical shift changes of both molecules in mixed solution as a function of concentration and temperature, as in previous work on complexation of DAU with NOV [21]. However, unlike the previous work for the NOV–DAU system [21], NOE/ROE data could not be used for structural studies of the hetero-complexes, because no intermolecular cross-peaks were observed in 2D-NOESY and -ROESY spectra of either DAU–AMD or NOV–AMD mixed solutions, even at the highest initial concentrations of the drugs studied. Negligible intensities of intermolecular NOE contacts may be due to a combination of the limited solubility of AMD and NOV in aqueous salt solution (0.1 M phosphate buffer) and the rather large number of different types of stacks in terms of their sizes and 1:1 hetero-interfaces in the equilibrium distribution of complexes between aromatic drugs in aqueous solution [14]. An example of the changes in chemical shifts with concentration is given in Fig. 2a for one of the systems studied, DAU–AMD, in which the concentration of AMD is constant (0.26 mM) and the concentration of DAU varied. It is observed in Fig. 2a that all the protons of AMD and DAU (except H10*a*) move to low frequency with an increase of concentration of DAU in the mixed solution, consistent with an increasing proportion of stacked complexes of these aromatic molecules. Qualitative comparison of the concentration dependences of proton chemical shifts of AMD and DAU in the hetero-association experiments of the antibiotics (Fig. 2a) with those for their self-association [10,12] indicate that there is greater shielding of the protons of molecules in the hetero-complexes compared with those observed for self-association. The different chemical shift behavior of H10*a* of DAU may be a result of the substantially higher shielding of the H10*a* proton in the AMD–DAU complex compared with that in the DAU dimer and/or by the shift of the molecular equilibrium of associated forms of DAU with an increase of its concentration in solution (see below). An example of the temperature dependence of chemical shifts of DAU and AMD in mixed solution is shown in Fig. 2b where all signals move to high frequency at higher temperatures as molecular complexes dissociate.

3.2. Hetero-association model

The experimental NMR data were analyzed by a statistical thermodynamical model of molecular hetero-association of two component molecules A and P [18], using a dynamic equilibrium that includes indefinite self-association of both A and P as well as indefinite hetero-

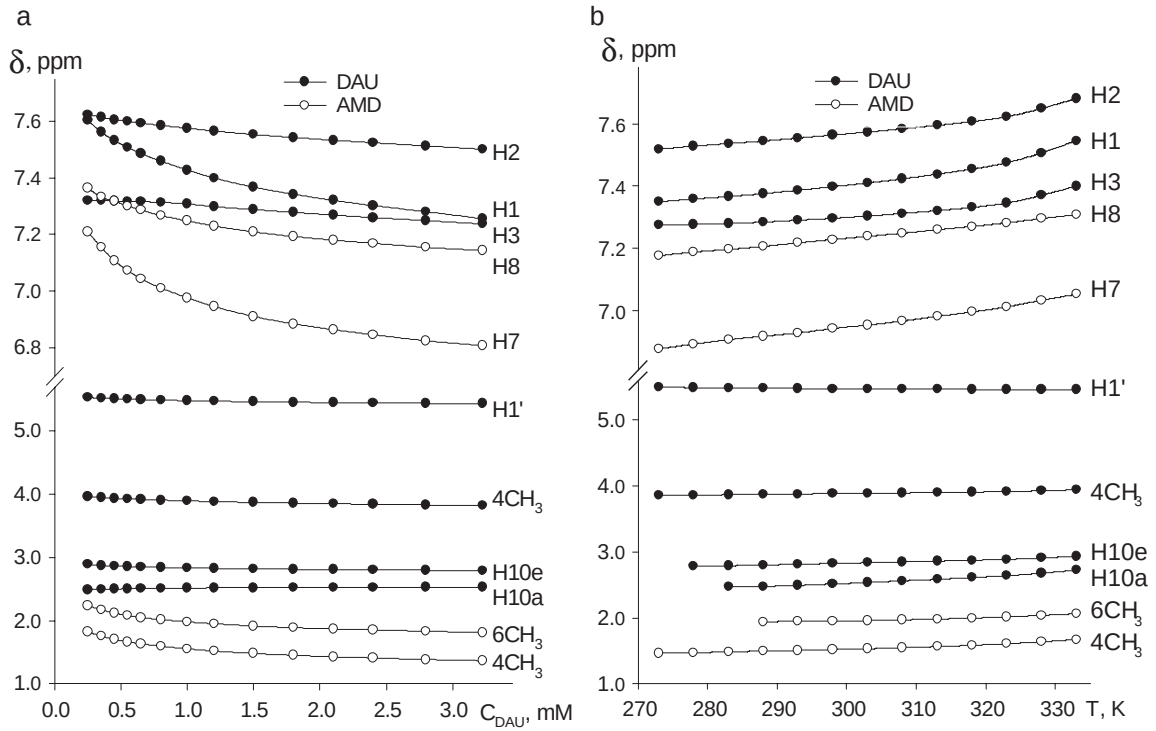

Fig. 2. Dependence of proton chemical shifts of aromatic drugs in DAU–AMD mixtures in 0.1 M phosphate buffer, pD=7.1: a) on concentration of DAU (T=298 K, $C_{\mathrm{AMD}}=p_0$=0.26 mM); b) on temperature at $C_{\mathrm{DAU}}=a_0$=1.20 mM, $C_{\mathrm{AMD}}=p_0$=0.26 mM.

association reactions of different types, as shown in the following reactions scheme (1):

$$\begin{aligned} &A_1 + A_i \overset{K_{\mathrm{A}}}{\leftrightarrow} A_{i+1}(a), \qquad P_1 + P_j \overset{K_{\mathrm{p}}}{\leftrightarrow} P_{j+1}(b),\\ &A_i + P_j \overset{K_{\mathrm{h}}}{\leftrightarrow} A_iP_j(c), \qquad P_jA_i + P_l \overset{K_{\mathrm{h}}}{\leftrightarrow} P_jA_iP_l(d),\\ &A_k + P_jA_i \overset{K_{\mathrm{h}}}{\leftrightarrow} A_iP_jA_k(e) \end{aligned} \tag{1}$$

where A_1 and P_1 correspond to the monomers of A and P component drugs and A_i, A_k, P_j and P_l are the aggregates containing i, k monomers of A and j, l monomers of the P component, respectively. Equilibrium association constants for the reactions of self-association of A (K_{A}) and P (K_{P}) components and their hetero-association (K_{h}) in the statistical thermodynamical model [18] are assumed to be independent of the number of molecules in the aggregates and complexes. However, sedimentation studies of aqueous solutions of the antibiotic actinomycin D [32] confirm that self-association aggregates of AMD exist predominantly as dimers and that higher order aggregates hardly form in solution even at drug concentrations close to saturation. It is likely that formation of higher order aggregates for the antibiotic AMD in solution is unfavorable for steric reasons caused by the two bulky pentapeptide side chains (Fig. 1). Hence, for self-association of AMD and its hetero-association with DAU and NOV, the dimer model was used to analyze the experimental NMR data, i.e. in reactions (1) j, l=1,2 for the formation of hetero-complexes (reactions 1c, 1d and 1e). In reactions (1) A_1 and P_1 correspond to monomers of NOV/DAU and AMD, respectively, A_i, A_k, P_j, P_l represent self-aggregates containing i,k molecules of NOV/DAU and j, $l \leq 2$ molecules of AMD.

Taking into account the mass conservation and the mass action laws for reactions (1), the dependence of the observed proton chemical shifts of NOV/DAU on the drug concentration in the mixed solution can be written in the form [18] modified to take into consideration the dimer association of AMD:

$$\begin{aligned} \delta_A = \frac{a_1}{a_0}\Bigg[&\delta_{m\mathrm{A}}\left(2(1+K_{\mathrm{A}}a_1) - \frac{1}{(1-K_{\mathrm{A}}a_1)^2}\right)\\ &+ 2\delta_{d\mathrm{A}}\left(\frac{1}{(1-K_{\mathrm{A}}a_1)^2} - 1 - 1K_{\mathrm{A}}a_1\right)\\ &+ \delta_{h\mathrm{A}}\frac{K_{\mathrm{h}}\left(p_1+K_{\mathrm{h}}p_1^2\right)}{(1-K_{\mathrm{A}}a_1)^2}\left(1+\frac{K_{\mathrm{h}}\left(p_1+K_{\mathrm{h}}p_1^2\right)}{2}\right.\\ &\left.+\frac{K_{\mathrm{h}}a_1}{1-K_{\mathrm{A}}a_1}\right)\Bigg] \end{aligned} \tag{2}$$

and for AMD protons:

$$\begin{aligned} \delta_p = \frac{p_1}{p_0}\Bigg[&\delta_{m\mathrm{P}} + 2\delta_{d\mathrm{P}}K_{\mathrm{P}}p_1 + \delta_{H\mathrm{P}}\frac{K_{\mathrm{h}}a_1\left(1+2K_{\mathrm{p}}P_1\right)}{1-K_{\mathrm{A}}a_1}\\ &\times\left(1+\frac{K_{\mathrm{h}}a_1}{2(1-K_{\mathrm{A}}a_1)}+K_{\mathrm{h}}\left(p_1+K_{\mathrm{p}}P_1^2\right)\right)\Bigg] \end{aligned} \tag{3}$$

where a_0, p_0 and a_1, p_1 are the initial and monomer concentrations of NOV/DAU and AMD, respectively; $\delta_{m\mathrm{A}}$, $\delta_{d\mathrm{A}}$, $\delta_{h\mathrm{A}}$ and $\delta_{m\mathrm{P}}$, $\delta_{d\mathrm{P}}$, $\delta_{h\mathrm{P}}$ are the corresponding values of

Table 1
Calculated values of hetero-association parameters of anticancer antibiotics in 0.1 M phosphate buffer, pD=7.1, T=298 K

DAU+AMD

Protons A (DAU)	δ_{hA}, ppm	δ_{dA}, ppm	δ_{mA}, ppm	Protons P (AMD)	δ_{hP}, ppm	δ_{dP}, ppm	δ_{mP}, ppm	K,·10^3 M^{-1}
H2	7.42	7.53	7.83	H8	7.07	7.41	7.51	K_A=0.72±0.13
H1	7.20	7.33	7.78	H7	6.74	7.41	7.51	K_P=1.42±0.13
H3	7.18	7.26	7.55	$6CH_3$	1.75	2.39	2.60	K_h=2.75±1.10
H1′	5.56	5.43	5.52	$4CH_3$	1.27	1.69	2.28	
$4OCH_3$	3.89	3.83	4.02					
H10*e*	2.61	2.86	3.05					
H10*a*	2.25	2.64	2.81					

NOV+AMD

Protons A (NOV)	δ_{hA}, ppm	δ_{dA}, ppm	δ_{mA}, ppm	Protons P (AMD)	δ_{hP}, ppm	δ_{dP}, ppm	δ_{mP}, ppm	K,·10^3 M^{-1}
H6/7	7.46	7.18	7.68	H8	7.36	7.40	7.51	K_A=28.90±9.30
H2/3	6.86	6.91	7.30	H7	7.32	7.40	7.51	K_P=1.42±0.13
H11	3.87	3.69	3.96	$6CH_3$	2.25	2.46	2.60	K_h=2.50±1.00
				$4CH_3$	1.75	1.90	2.28	

proton chemical shifts of NOV/DAU and AMD in the monomer, dimer forms and in the hetero-complexes. Values of the proton chemical shifts δ_{mA}, δ_{dA}, δ_{mP}, δ_{dP} and the equilibrium constants K_A, K_P for the interacting molecules have been determined previously from independent experiments on investigations of the self-association of the drugs under the same experimental conditions [10–12]. The computational procedure for calculation of the hetero-association parameters of the drug molecules is described in detail elsewhere [11] and the calculated parameters for formation of hetero-association complexes of DAU–AMD and NOV–AMD at T=298 K are summarized in Table 1.

3.3. Thermodynamics

The thermodynamical parameters of hetero-association, ΔH^0_{het} and ΔS^0_{het}, of the aromatic drugs (AMD–DAU and AMD–NOV) have been determined from measurements of the proton chemical shifts of molecules in the mixed solution as a function of temperature (Fig. 2b), using the additive model for observed proton chemical shifts and the van't Hoff's formalism developed previously [12].

Eqs. (2) and (3) were used for calculations of thermodynamical parameters in which the influence of temperature on the values of δ(T) is determined by the temperature dependence of the equilibrium constants of self- and hetero-association of molecules in solution

$$K_i(T) = \exp\left(\Delta S_i^0/R - \Delta H_i^0/RT\right) \quad (4)$$

To a first approximation, the values of ΔH_i^0 and ΔS_i^0 do not change substantially with temperature in the range studied, as shown by the linear van't Hoff plots in Fig. 3. The derived mean values of the thermodynamical parameters of hetero-association of AMD with DAU or NOV are summarized in Table 2. For comparison purposes, the ΔH^0_{het} and ΔS^0_{het} values obtained previously for the NOV–DAU system under the same experimental conditions [21] are also presented in Table 2.

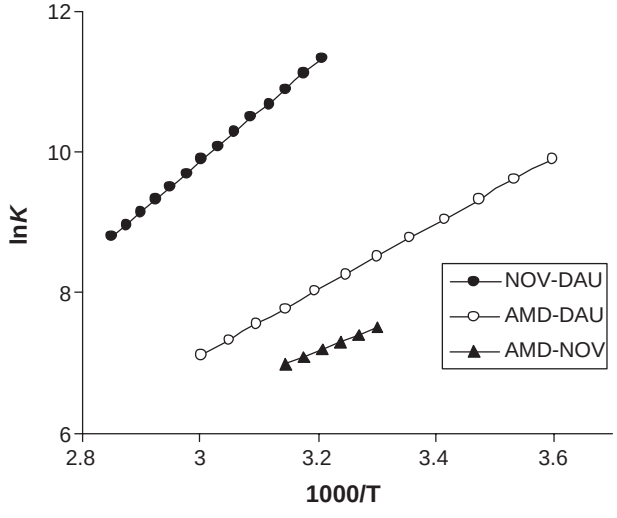

Fig. 3. Linear plots of ln$K$ against 1000/T according to Eq. (4) for hetero-association of DAU–AMD and NOV–AMD. Comparable data for NOV–DAU taken from previous work [21].

4. Discussion

4.1. Hetero-association parameters

It can be seen in Table 1 that the calculated magnitudes of the hetero-association constants (K_h) for the NOV–AMD

Table 2
Thermodynamical parameters of hetero-association of aromatic anticancer antibiotics in aqueous solution (0.1 M phosphate buffer, pD=7.1, T=298 K)

System	$-\Delta G^o_{het}$, kJ/mol	$-\Delta H^o_{het}$, kJ/mol	$-\Delta S^o_{het}$, J/mol·K
NOV+AMD	19.4±0.8	28±7	30±8
DAU+AMD	19.6±0.8	39±5	58±12
DAU+NOV[a]	23.9± 0.2	59±6	95±10

[a] Data taken from Ref. [21].

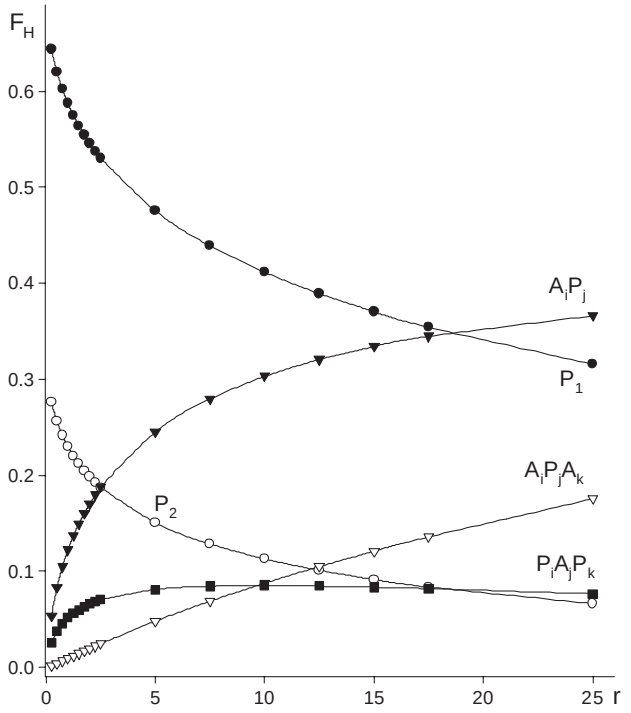

Fig. 4. Relative content (F_H) of self-aggregates of actinomycin D (P) and its hetero-complexes with novatrone (A) as a function of the ratio of the initial concentrations of NOV (a_0) to AMD (p_0), $r=a_0/p_0$, $p_0=0.4$ mM=const., $T=298$ K.

and DAU–AMD systems are similar, in which K_h for NOV–AMD is intermediate between the values of the self-association constants of the individual aromatic molecules but K_h for the DAU–AMD system is greater than the self-association constants of the component molecules. It was found previously that, in those cases where the hetero-association constants of the interacting molecules are greater than the self-association constants, there is additional stabilisation due to possible formation of intermolecular hydrogen bonds between donor–acceptor groups of aromatic chromophores in the stacked hetero-complexes [18,21]. Hence, it is likely that in the DAU–AMD system intermolecular hydrogen bonds also give some contribution to stabilisation of the hetero-complex of the aromatic drugs. However, for the NOV–AMD system, where the K_h value is intermediate between the values of AMD and NOV self-association constants (Table 1), hydrogen-bond formation in the NOV–AMD hetero-complex may be considered to be less likely.

Using the values of equilibrium constants of self-association of actinomycin D [12], daunomycin [10] and novatrone [11] and their hetero-association constants (Table 1), the relative content of each type of molecular complex in mixed solution has been calculated as a function of r ($=a_0/p_0$, the ratio of the initial concentrations of the interacting drugs). The results for the NOV–AMD system are presented, as an example, in Fig. 4 and the results for DAU–AMD have an overall similar dependence. It is seen from Fig. 4 that there is an increase in content of the hetero-complexes (A_iP_j) and ($A_iP_jA_i$) between AMD and NOV with increasing concentration of novatrone in solution (the concentration of AMD being kept constant at 0.4 mM). At $r>12$ the sum of all the hetero-complexes becomes predominant in the mixed solution (Fig. 4). Similar analysis of the results for the DAU–AMD system in this work shows that the predominance of the hetero-complexes in the mixed solution occurs at $r>2$ for DAU–AMD, in contrast to that for DAU–NOV ($r>0.3$) measured previously [21]. These results indicate that the contributions of hetero-complexes to the general equilibrium predominate at quite different values of r in the mixed solutions of anticancer drugs. It follows that anticancer drugs have quite different affinities for formation of complexes with other aromatic antibiotics in aqueous solution, which may result in modifying the effects of their medico-biological properties.

4.2. Structures of DAU–AMD and NOV–AMD hetero-association 1:1 complexes in aqueous solution

A comparison of the induced proton chemical shifts of drugs in the hetero-complex $\Delta\delta_h(=\delta_m-\delta_h)$ and in self-associated dimers, $\Delta\delta_d=\delta_m-\delta_d$ (Table 1) provides some preliminary information about the structures of the 1:1 DAU–AMD and NOV–AMD hetero-complexes formed in aqueous solution. Thus, an approximately proportional distribution of proton shielding (i.e. $\Delta\delta_h$ and $\Delta\delta_d$ ratios for different non-exchangeable protons) is observed in both the hetero-complex and dimers of AMD and DAU but the value of $\Delta\delta_h$ is essentially higher in the DAU–AMD complex compared to $\Delta\delta_d$ in DAU–DAU and AMD–AMD dimers [10, 12]. Hence, it is likely that the distance between the aromatic chromophores in the 1:1 DAU–AMD hetero-complex is smaller than in AMD and DAU dimers [10,12]. On the other hand, such a situation is not observed for NOV–AMD complexation (Table 1) in comparison with the

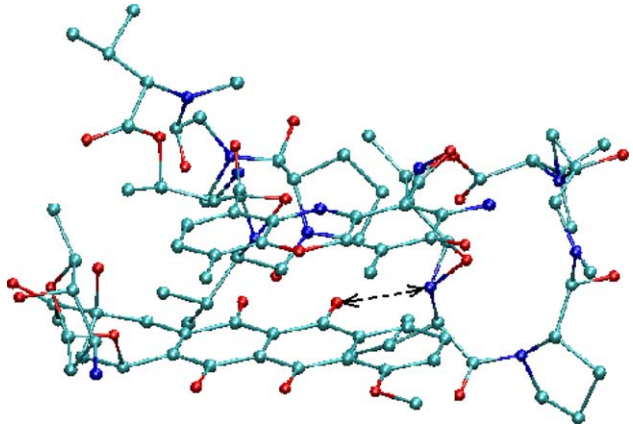

Fig. 5. The molecular mechanics calculated spatial structure of the 1:1 DAU–AMD hetero-complex using NMR data. An intermolecular hydrogen bond (shown by the arrow) may be formed between O12 of ring C of DAU and the NH group of D–Val of the cyclopentapeptide side chain, which is attached to carbon C1 of the AMD chromophore.

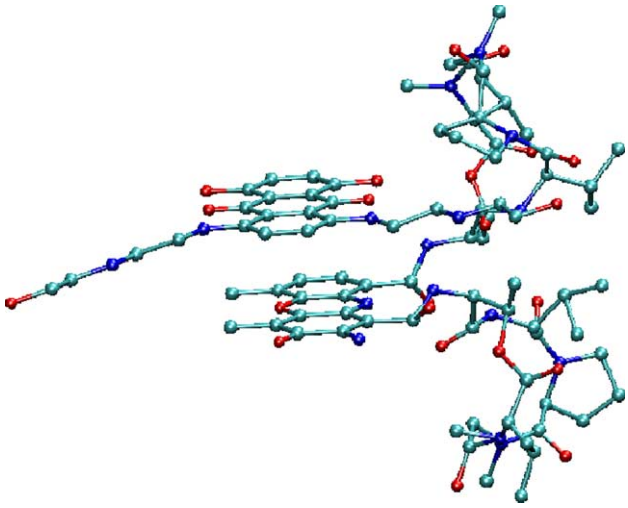

Fig. 6. The molecular mechanics calculated spatial structure of 1:1 NOV–AMD hetero-complex using NMR data.

dimers of NOV–NOV [11] and AMD–AMD [12]. It should be noted that the behavior of H10*a* (Fig. 2a), which is different to the other protons, results from a rather large shielding for this proton in the DAU–AMD hetero-complex ($\Delta\delta_h$) compared with that in the DAU dimer ($\Delta\delta_d$) (Table 1, $\Delta\delta_h = 0.56$ ppm and $\Delta\delta_d = 0.17$ ppm) and the large distribution of associated molecular complexes of DAU as a function of its concentration in the mixed solution (Fig. 4). The values of the limiting proton chemical shifts δ_h obtained for AMD, DAU and NOV (Table 1) were used to determine the most favorable structures of the 1:1 DAU–AMD and NOV–AMD hetero-complexes in aqueous solution by comparison of the induced proton chemical shifts, $\Delta\delta_h$, and their theoretical values derived from quantum-mechanical calculations of iso-shielding curves for AMD, NOV and DAU [33]. Thus, the values of induced proton chemical shifts of the molecules in the DAU–AMD hetero-complex (Table 1) show that the quinone ring of the AMD chromophore containing the $4CH_3$ protons is situated above the aromatic ring D of daunomycin containing protons *H*1, *H*2 and *H*3.

The most probable spatial structures of the 1:1 DAU–AMD and NOV–AMD hetero-complexes in aqueous solution, calculated by the method of molecular mechanics are presented in Figs. 5 and 6, respectively. In the calculated structures the planes of the chromophores of AMD and DAU molecules in the 1:1 hetero-complex (Fig. 5) are parallel to each other at a distance of about 0.32 nm, whereas in the NOV–AMD hetero-complex (Fig. 6) they are situated 0.34 apart, similar to that observed in dimers of DAU [10] and NOV [11]. A large overlap of the aromatic parts of the chromophores has been found for both DAU–AMD and NOV–AMD hetero-complexes, indicating substantial stacking interactions of the drug molecules. The calculated structure of the 1:1 DAU–AMD complex also indicates that a hydrogen bond (shown by the dotted line in Fig. 5) could be formed between O12 of ring C of DAU and the NH group of D–Val of the pentapeptide side chain ring of AMD, which is attached to carbon C1 of the chromophore.

It should be noted that the calculated structures of the 1:1 DAU–AMD (Fig. 5) and NOV–AMD (Fig. 6) complexes are only approximate but they are consistent with both the limiting proton chemical shifts for these molecular systems and the minimum values of their potential energies determined by the methods of molecular modeling.

4.3. Thermodynamical parameters of hetero-association of anticancer antibiotics in aqueous solution

Dispersive van der Waals interactions are characterized both by negative enthalpy and entropy [34]. Hence, the rather large negative values of enthalpy and entropy of hetero-association observed for DAU–AMD and NOV–AMD (Table 2) indicate that dispersive interactions play an essential role in the formation of hetero-complexes of these molecules. Quantitative analysis of the thermodynamic parameters of formation of hetero-complexes is consistent with the provisional conclusion that the DAU–AMD hetero-complex in aqueous solution may also be stabilized by intermolecular hydrogen bonds. It is seen from Table 2 that the enthalpies and entropies of complexation of both DAU–AMD and DAU–NOV [21], where the hetero-association complex is stabilized by hydrogen bonds, are substantially higher in absolute values compared with that for NOV–AMD complexation, i.e. the difference in ΔH for DAU–AMD complexation compared with NOV–AMD is similar to the estimated magnitude of enthalpy of hydrogen bond formation in aqueous solution from −8 up to −13 kJ/mol [35].

It is concluded that aromatic antitumour drugs, such as actinomycin D, novatrone and daunomycin, form energetically rather stable hetero-association complexes in aqueous solution, which may subsequently effect their medical and biological activity as they are known to be DNA intercalators and exert their biological activity by direct interaction with nuclear DNA [36]. Such investigations are important for elucidation of the interrelations of antitumour antibiotics and their likely compatibility in combination chemotherapy.

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