Ultrasonic absorption frequency dependence of two widely used anti-cancer drugs: doxorubicin and daunorubicin

D. B. Tata†*, G. Hahn† and F. Dunn‡

†Department of Radiation Oncology, Stanford School of Medicine, Stanford University, Stanford, California 94305, USA
‡Bioacoustics Research Laboratory, University of Illinois, Urbana, Illinois 61801, USA

Received 10 November 1992; revised 15 March 1993

Low intensity ultrasound (approximately 10^{-6} W cm^{-2}) in the frequency range 0.5–6.0 MHz was employed to investigate the ultrasound absorption properties of doxorubicin (DOX) at several temperatures. At physiological temperatures, we found enhanced ultrasound absorption from DOX, and its closely related analogue daunorubicin (DNR), in the upper kilohertz frequency range. The findings do not conform to classical theory of ultrasound absorption, thus suggesting an ultrasound coupling with the drug molecules via structural and/or chemical relaxation processes. The absorption spectra are analysed from the point of view of the non-classical theory of sound absorption due to physical and/or chemical relaxations. Only one spectral difference between the two anti-cancer agents is observed, around 2 MHz, and may be attributed to the sole difference in the chemical make-up of the side chain of the two antibiotics.

Keywords: absorption properties; anti-cancer drugs; doxorubicin; daunorubicin

There have been recent reports on the enhancement of the therapeutic efficacy of a widely used anti-cancer agent, namely doxorubicin (DOX), when exposed to therapeutic intensity ultrasound, i.e. 1–3 W cm^{-2} (see References 1–3), and even at sub-therapeutic intensity levels[4]. The cause for such enhancements in cell killing appears to be due to non-thermal processes. *In vitro* experiments have implicated enhanced production of superoxides when clinical levels of DOX were exposed to therapeutic intensity ultrasound through studies involving superoxide dismutate, and the reduction of cytochrome c (see Reference 5). *In vivo* combination treatments of DOX and therapeutic intensity level ultrasound have found delays in tumor growth[5].

In order to understand the underlying non-thermal mechanisms involved in leading to the observed enhanced cell killing, it becomes necessary first to investigate and characterize the dynamics of such anti-cancer drug interaction with the local environment. One common method of interrogating systems in thermodynamic equilibrium is to apply small harmonic perturbations of a thermodynamic parameter, such as temperature or pressure, and to monitor the system's relaxation response[6]. In the present investigation, continuous low intensity ultrasound (approximately 10^{-6} W cm^{-2}) was employed in a first attempt to understand the dynamical interactions of two clinically used anthracycline drugs, namely DOX and daunorubicin (DNR), with their local aqueous environment, at several temperatures via ultrasound absorption spectroscopy.

Ultrasound absorption spectroscopy is a powerful tool that has been employed in the past for studying various protein and lipid conformational changes[7,8] and in the determination of the kinetic rate constants of a variety of reactions in solutions, including those involving protonation and deprotonation[9]. The experimental data presented in this communication show that ultrasound absorption spectroscopy can possibly be used as a tool to aid in the identification of drugs whose cytotoxic activity is enhanced by ultrasound.

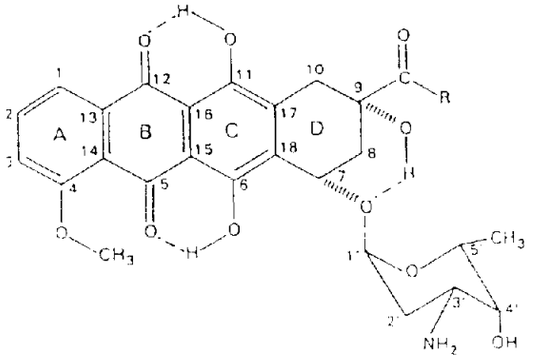
The dimensionless absorption per wavelength, $\alpha\lambda$, is employed as the quantitative measure of the diminution of the acoustic pressure and intensity as the wave travels a distance of one wavelength, λ.

Materials and methods

Doxorubicin was obtained from Adria Laboratories and Daunorubicin from Sigma Chemical Company. The chemical structure of these two anthracycline antibiotics is shown in *Figure 1*. Both drugs were dissolved in 0.9% NaCl saline buffer solution and aliquots were made under sterile conditions and kept frozen in the dark until the

*Present address: Department of Pharmacology, University of Vermont, Burlington, Vermont 05405, USA

0041-624X/93/060447-04



Side Chain R: DOX = CH_2OH DNR= CH_3

Figure 1 Chemical structure of DOX and DNR

time of measurement. The ultrasound absorption measurements performed in this investigation on drug suspensions were made at a fixed drug concentration of 100 μg ml^{-1} within a conventional Eggers and Funck type cylindrical resonator[10] within the operational frequency range of 0.5–6 MHz and in the temperature range of 5–37°C for DOX and at 37°C for DNR. All absorption measurements were repeated three different times for all temperatures investigated herein. The sensitivity of the acoustic resonator was determined through testing the accuracy of the known difference in sound velocity between degassed distilled H_2O and 0.9% NaCl solution at room temperature at several frequencies. The operational elements of the acoustic resonator and measurement procedures are reported elsewhere[8]. Briefly, the end walls of the resonant cavity are formed by two identical piezoelectric quartz transducers separated a distance d (= 5.5 cm) by a hollow Plexiglas cylinder, providing a sample volume of approximately 3 ml. One transducer is excited continuously by a Hewlett-Packard synthesized signal generator, at a predetermined frequency, and produces longitudinal plane waves in the fluid medium within the cavity while the other transducer acts as the receiver. The amplitude of the resulting standing wave at the receiving transducer surface is monitored by a Hewlett-Packard 8552A, 8553B spectrum analyser. The amplitude of the standing wave is a maximum at resonance frequencies when the standing wave boundary conditions are fulfilled, i.e. when d is an odd number of half-wavelengths[8].

The $\alpha\lambda$ for the nth resonance is directly related to the half-power bandwidth of the resonance Δf_n, i.e. $\alpha\lambda = \pi\Delta f / f_n$ (see Reference 10). For the drug concentration study reported here, it is assumed explicitly that the measured $\alpha\lambda$ of the drug suspensions are comprised of an additive sound absorption contribution from the drug molecules and from the solvent medium as $(\alpha\lambda)_{drug} = (\alpha\lambda)_{drug\ suspension} - (\alpha\lambda)_{solvent}$. Consequently, from the above assumption, the excess absorption per wavelength due to the presence of the drug is given by

$$(\alpha\lambda)_{drug} = \pi(\Delta f_{solution} - \Delta f_{solvent})/f_n \qquad (1)$$

Results

The concentration independent DOX $\alpha\lambda$ frequency dependence is shown for several temperatures in *Figure 2*, and is seen to exhibit a strong temperature dependence. At physiological temperature (37°C) there is evidence for the existence of a prominent absorption peak situated in the upper kilohertz frequency range, 350 kHz to 500 kHz, which is below that of the acoustic resonator's operational range (governed by the geometrical boundary conditions). In addition to the prominent peak, there is evidence of a shoulder situated in the neighbourhood of 1 MHz and a small secondary peak around 2 MHz. The excess absorption frequency characteristics of DOX and DNR are compared at 37°C in *Figure 3*. The ultrasound absorption spectral differences between DOX and DNR are computed and shown in *Figure 4*. Throughout the investigated frequency range DOX is noted to absorb greater ultrasound energy than DNR. In the lower frequency regions between 0.5 MHz to 1.5 MHz, both DOX and DNR exhibit similar monotonic decreases in absorption and a shoulder around 1 MHz. A salient difference between the two sister compounds appears beyond 1.5 MHz where the DNR absorption spectrum continues to decrease smoothly, while the DOX absorption spectrum is seen to increase into a secondary peak around 2 MHz. All

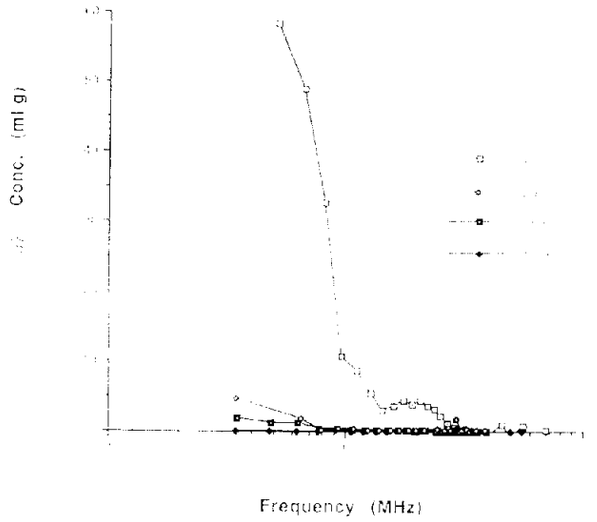


Figure 2 Concentration independent DOX ultrasound absorption at several temperatures. [DOX] = 100 μg ml^{-1}

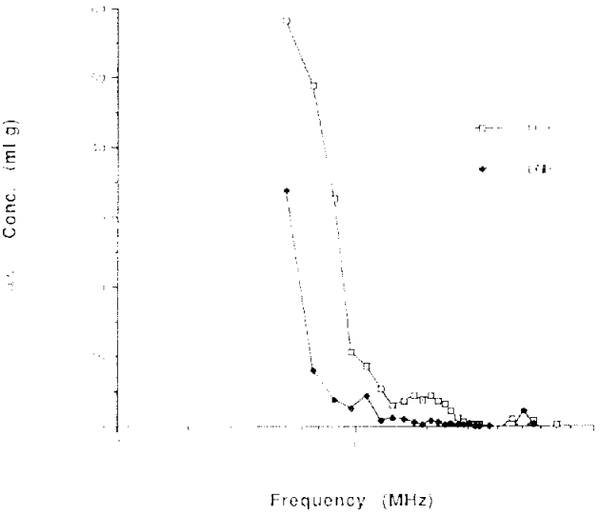


Figure 3 Concentration independent DOX and DNR ultrasound absorption at 37 C. [DOX] = [DNR] = 100 μg ml^{-1}

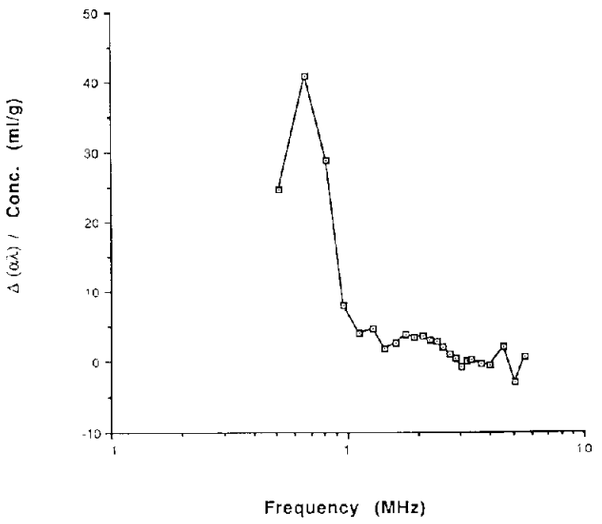


Figure 4 Concentration independent ultrasound absorption difference between DOX and DNR at 37°C. [DOX] = [DNR] = 100 μg ml^{-1}

spectral features, exhibited in the figures, were observed to be present in the three repeated measurements, at all temperatures reported herein.

Discussion

The mechanical energy in the propagating pressure wave is absorbed by the drug suspension under study by two classes of mechanisms, namely the classical absorption processes and the physical relaxation process[9]. Classical absorption arises as a consequence of the suspension having a finite viscosity and a finite thermal conductivity, which results, respectively, in shearing motions predominantly between the solvent molecules and in thermal energy being transported along temperature gradients. Non-classical absorption mechanisms result, for example, when the chemical or structural equilibrium is perturbed by the changes in pressure and temperature induced by the sound wave. The two classical and many possible non-classical absorption processes contribute to the pressure amplitude and intensity absorption coefficient α, respectively, in $p(x) = p_0 \exp(-\alpha x)$ and $I = I_0 \exp(-2\alpha x)$. The two classes of sound absorption processes are generally assumed to combine linearly as

$$\alpha = \alpha_{\text{classical}} + \alpha_{\text{non-classical}} \tag{2}$$

Acoustic theory of sound absorption in liquid media predicts the $\alpha\lambda$ behaviour with frequency, for both classes of processes. For classical processes, the solvent's $\alpha\lambda$ is directly proportional to the ultrasound frequency in the 10^6 Hz–10^7 Hz region, since the relaxations due to viscous mechanisms of a water-based medium are known to occur at extremely high relaxation frequencies[9] (around several gigahertz). For a relaxation process occurring in the region of measurement, the frequency dependence is more complex and depends also upon the characteristic relaxation time, τ, of the process, i.e. $\alpha\lambda \sim (\omega\tau)/\{1 + (\omega\tau)^2\}$. Typically, the sound absorption spectra of solutions of biomolecules are resolved in terms of several independent single relaxations, each having their characteristic relaxation time τ.

The measured frequency dependences of DOX and DNR clearly do not conform to the classical theory of sound absorption. As stated above, in view of the non-classical theory, the $(\alpha\lambda)_{\text{drug}}$ includes contributions of one or more processes such as structural and/or chemical relaxation, each having their characteristic time constants τ_i and their maximal relaxation strengths $(\alpha\lambda_i)_{\text{max}}$. Consequently

$$\alpha\lambda = \sum_i 2(\alpha\lambda_i)_{\text{max}} \frac{(\omega\tau_i)}{1 + (\omega\tau_i)^2} \tag{3}$$

is the expected frequency dependence of the non-classical absorption. After a lengthy manipulation procedure, the structural and chemical relaxation strengths of the 'solute' molecules are formulated to depend quadratically upon the differences in molar volume and enthalpy between the final (product) and initial (reactant) states of the induced transition, and directly proportional to the product of the (equilibrium) fractional population of molecules occupying the states of transition[6,9].

Although it is difficult to discern the type(s) of mechanism(s) involved in producing the ultrasound absorption profiles, the time constants of the process giving rise to the prominent peak in DOX at physiological temperature can be estimated. This is accomplished by assuming explicitly a single two-state transition process leading to a significant sound absorption in the lower frequency range, i.e. Equation (3) becomes

$$\alpha\lambda = 2(\alpha\lambda)_{\text{max}} \frac{(\omega\tau_i)}{1 + (\omega\tau_i)^2} \tag{4}$$

Evaluation of the time constant is accomplished by observing that the points of inflection can be obtained from the data of *Figures 2* and *3*. Thus, the second derivative of $\alpha\lambda$ with respect to frequency, in Equation (4), is set equal to zero. The main peak's point of inflection can be determined from the absorption plot and fitted into the second derivative equation equated to zero. This condition is found to be fulfilled when $\omega\tau = f_{\text{infl}}/f_{\text{relax}} = +\sqrt{3}$. Consequently, $f_{\text{relax}} = f_{\text{infl}}/\sqrt{3}$.

$(\alpha\lambda)_{\text{max}}$ can now be determined from the known value of $(\alpha\lambda)$ at the point of inflection and through Equation (4), namely $(\alpha\lambda)_{\text{max}} = (\alpha\lambda)_{\text{infl}}/\sqrt{3/4}$. From the DOX absorption plot (*Figure 3*), the point of inflection is determined to be situated around 0.7 MHz and this yields $f_{\text{relax}} \sim 0.4$ MHz and $(\alpha\lambda)_{\text{max}}/\text{conc.} = 60$. The DNR absorption data do not reveal a point of inflection in the prominent peak (*Figure 3*); presumably it lies at a frequency below that of the resonator operation range.

It is difficult to comment on the relaxation strength or the frequencies of relaxation in the low temperature studies reported herein for DOX, due to a lack of discernible points of inflection from the absorption spectra. Further investigative efforts with specially constructed low-frequency resonators should yield fruitful information on the acoustical and kinetic parameters.

The difference in ultrasound absorption between the two closely related analogues near 2 MHz presumably results due to the one single difference within their side chains (*Figure 1*). DOX has a ketol side chain which is known to play a significant role in facilitating non-enzymatic production of superoxides in the presence or absence of transition metals. However, DNR has a ketone side chain, and is known to reduce molecular or absence of transition metals. However, DNR has a

oxygen poorly, even when strongly bound to transition metals[11]. If the oxidation of the ketol side chain is enhanced through DOX configurational changes, then it is reasonable to hypothesize that ultrasound can be utilized as a therapeutic tool to induce molecular configurational changes, and thus to increase the rate of electron transfer to molecular oxygen. The frequency range in which there is maximum coupling of the pressure wave with the drug constituents can be expected to be determined through ultrasound absorption spectroscopy. Our data and model presented above may, in part, explain our previous *in vitro* finding of (therapeutic intensity) 1.5 MHz ultrasound enhanced cytotoxicity of DOX, but not that of DNR in Chinese hamster ovary cells.

Ultrasound absorption studies from a number of different biomolecules have shown similar absorption peak frequency locations (i.e. well below the MHz range) to the prominent peaks found in this study, which are believed to arise through conformational changes induced via proton transfer reactions of various amino acid residues[12,13]. In view of this, we hypothesize that the main prominent peak from DOX and DNR may be attributed to a protonation↔de-protonation scheme at various groups on the drug molecules, particularly, at the amine on the sugar, and between the carbonyl and hydroxyl groups of benzo and hydro quinone moieties, respectively. It is equally appealing to hypothesize that the low frequency prominent peaks may be attributed to the ultrasound coupling to the drug's cyclic chemical reduction and auto-oxidation pathway. The anti-tumour activity of these agents has been claimed to be, in part, due to continuous reduction and auto-oxidation of their quinone moieties, which generates significant amounts of superoxides, hydrogen peroxides, and hydroxyl radicals[14–17]. The validity of this hypothesis can potentially have far reaching clinical consequences whereby ultrasound could be utilized as a tool to enhance production rates of reactive oxygen species (such as superoxides, hydroxyl radicals and hydrogen peroxides) from selective redox cycling anti-cancer drugs at their natural redox cycling frequencies. Depending on the validity of the above hypothesis, ultrasound absorption spectroscopy could then play an active role in screening out redox cycling drugs and determine their natural redox cycling frequencies. An exhaustive literature search has revealed an intriguing observation: out of the several anti-cancer drugs investigated with ultrasound, by and large, the ones which contain a quinone moiety, namely DOX, DNR, mitomycin c, and diaziquinone, have yielded positive enhancement in cytotoxicity. Preliminary low frequency ultrasound investigations at 0.5 MHz and at a therapeutic intensity level of 1 W cm^{-2} in the presence of DOX or Fe(III) bound DOX has revealed substantial generation rates of hydroxyl radicals over the duration of ultrasound irradiation[18].

Acknowledgements

D.B. Tata and G. Hahn gratefully acknowledge the partial support of this work by NIH grant CA 19386. F. Dunn gratefully acknowledges the partial support of this work by NIH grant CA 45689.

References

1 **Loverock, P., Ter Haar, G., Ormerod, M.G. and Imrie, P.R.** The effect of ultrasound on the cytotoxicity of adriamycin. *Brit J Radiology* (1990) **63** 542–546

2 **Saad, A.H. and Hahn, G.M.** Ultrasound enhanced toxicity on Chinese hamster ovary cells in vitro. *Cancer Research* (1989) **49** 5931–5934

3 **Yumita, N., Okumura, A., Nishigaki, R., Umemura, K. and Umemura, S.** The combination treatment of ultrasound and anti-tumor drugs on Yoshida sarcoma. *Japan J Hyperthermic Oncology* (1987) **3** 175–182

4 **Harrison, G.H., Balcer-Kubiczek, E.K. and Eddy, H.A.** Potentiation of chemotherapy by low-level ultrasound. *Int J Rad Biol* (1991) **59** 1453–1466

5 **Yumita, N., Okumura, A., Nishigaki, R. and Umemura, K.** The increase of generation of superoxide radicals and the inhibitory effect on Yoshida sarcoma of anthracycline antitumor agents by ultrasound. *J Japan Soc Cancer Therapeutics* (1989) **24** 63–68

6 **Eigen, M. and De Maeyer, L.** Relaxation methods. in *Techniques of Organic Chemistry* 8, Part 2 (Ed Weissberger, A.) Interscience, New York (1963)

7 **Strom-Jensen, P.** Ultrasonic absorption in solutions of proteins and peptides and in suspensions of liposomes. PhD Thesis, University of Illinois (1983)

8 **Tata, D.B. and Dunn, F.** Interaction of ultrasound and model membrane systems: Analyses and predictions. *J Phys Chem* (1992) **96** 3548–3555

9 **Matheson, A.J.** *Molecular Acoustics* Wiley, New York (1971)

10 **Eggers, F. and Funck, T.** Ultrasonic measurements with milliters liquid samples in the 0.5–100 MHz range. *Rev Sci Instru* (1973) **44** 969–977

11 **Gianni, L., Vigano, L., Lanzi, C., Niggeler, M. and Malatesa, V.** Role of daunosamine and hydroxyacetyl side chain in reaction with iron and lipid peroxidation by anthracyclines. *J National Cancer Institute* (1988) **80** 1104–1111

12 **Barnes, C., Evans, J.A. and Lewis, J.T.** Low frequency ultrasound absorption in aqueous solutions of hemoglobin, myoglobin, and bovine serum albumin: the role of structure and pH. *J Acoust Soc Am* (1988) **83** 2393–2404

13 **Strom-Jenson, P. and Dunn, F.** Ultrasonic absorption by solvent solute interactions in aqueous solutions of peptides and small proteins. *J Acoust Soc Am* (1984) **75** 960–966

14 **Sinha, B.K. and Mimnaugh, E.G.** Free radicals and anticancer drug resistance: oxygen free radicals in the mechanisms of drug cytotoxicity and resistance by certain tumors. *Free Radical Biol Med* (1990) **8** 567–581

15 **Keizer, H.G., Pinedo, H.M., Schuurhuis, G.J. and Joenje, H.** Doxorubicin (adriamycin): A critical review of free radical-dependent mechanisms of cytotoxicity. *Pharmac Ther* (1990) **47** 219–231

16 **Lusthof, K.J., De Mol, N.J., Richter, W., Janssen, L.H.M., Butler, J., Hoey, B.M., Verboom, W. and Reinhoudt, D.N.** Redox cycling of potential antitumor aziridinyl quinones. *Free Radical Biol Med* (1992) **13** 599–608

17 **Pratt, W. and Ruddon, R.W.** *The Anticancer Drugs* Oxford University Press, New York (1979)

18 **Tata, D.B., Wu, J. and Tritton, T.R.** Ultrasound enhanced generation of hydroxyl radicals from adriamycin and adriamycin + ferric iron. *Proceedings of the American Association for Cancer Research* (1993) to be published