Ultrasound-enhanced hydroxyl radical production from two clinically employed anti-cancer drugs, adriamycin and mitomycin C

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Continuous-wave 1 MHz ultrasound at the therapeutic intensity of 1 W cm\(^{-2}\) was found to enhance significantly the hydroxyl radical production from two clinically employed redox cycling drugs, viz. adriamycin (doxorubicin) and mitomycin C, with respect to the control drug-free insonicated phosphate buffer suspension. Benzoic acid (Bz) was employed as a sensitive chemical probe to detect hydroxyl radicals (HO\(^{\cdot}\)). Bz is initially non-fluorescent and upon aromatic hydroxylation becomes permanently fluorescent. A series of time course studies up to 30 min were performed on drug suspensions to characterize the HO\(^{\cdot}\) generation in the presence and absence of ultrasound at 37°C. Identical ultrasound treatments on non-redox cycling clinical drugs, 5-fluorouracil and methotrexate, did not yield any significant enhancement in the production of HO\(^{\cdot}\) in comparison to the drug-free insonicated phosphate buffer suspension. Ultrasound exposures of 30 min did not yield measurable changes in the chemical constitution of the four drugs as assessed through high-performance liquid chromatography. Identical ultrasound treatments at 3 MHz did not produce any HO\(^{\cdot}\) in the presence or absence of these four anti-cancer drugs. Free radical scavengers such as mannitol, superoxide dismutase, catalase and a transition metal chelating agent were employed independently to elucidate the chemical species and pathways involved in the production of the HO\(^{\cdot}\). The findings strongly implicate an active role of acoustically induced cavitation in potentiating redox cycling drugs via chemical reduction and, thereafter, production of the OH\(^{\cdot}\) via Fenton’s pathway.

Keywords: hydroxyl radicals; ultrasound, adriamycin; mitomycin C

There have been reports over the past decade regarding both in vitro and in vivo enhancements of anti-cancer drug activity due to exposure to treatments with therapeutic intensity level ultrasound\(^{1-5}\), typically 1–3 W cm\(^{-2}\). Of the several anti-cancer drugs studied with exposure to ultrasound, those containing a quinone moiety, namely adriamycin, daunorubicin, mitomycin C and diaziquinone, have been reported to yield positive enhancements in cytotoxicity as assessed through in vitro clonogenic assays, in vivo tumour size growth delays and extended days of specimen survival. The anti-tumour activities of these agents without ultrasound exposure have been claimed to be due, in part, to the redox cycling mechanism\(^{6-9}\), i.e. the ability of such compounds to be chemically or enzymatically reduced at their quinone groups and thereafter autoxidized from dissolved molecular oxygen. The redox cycling characteristics
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Figure 1 Ultrasound exposure set-up

![Diagram of ultrasound exposure set-up]

Figure 2 Calibration graph for OH$^-$ radicals produced by the Cs-137 source. Radiation rate = 16.27 rad s$^{-1}$, [Bz] = 700 μM

![Calibration graph for OH$^-$ radicals]

Fl. Units = (1.5808e-2 Fl. Units / Rad) (Dose) R$^2$ = 1.000

of these quinone-containing agents have been found experimentally to yield O$_2^-$, H$_2$O$_2$ and the highly reactive hydroxyl radicals, HO$^-$, due to the presence of biologically relevant transition metals such as Fe, Mg and Cu.

Recent improved engineering capabilities in focusing ultrasound in vivo have led to producing and controlling hyperthermia at the tumour site$^{10}$, resulting in marked enhancements in anti-cancer drug efficacy. It is intriguing to point out that even when tumour temperatures are closely monitored and maintained at normal body temperature during in vivo ultrasound treatments with anti-cancer drugs, marked enhancement in the drug’s cytotoxicity results$^{1,3}$. The non-thermal mechanism(s) which produce such enhancement in drug cytotoxicity is not known. Acoustically induced cavitation may be considered the dominant non-thermal mechanism responsible for promoting chemical reactions receiving ultrasound exposure$^{11}$. The chemical products from the acoustically induced gaseous bubble growth and collapse within aqueous media have been analysed with ESR$^{12,13}$ and fluorescent spectroscopic probes, such as benzoic acid$^{14}$, and it was found that the resulting action of acoustic cavitation led to the production of hydrogen atoms and hydroxyl radicals, due to complete dissociation of water vapour within the highly localized cavities. Thus, it is appealing to hypothesize that ultrasound-enhanced cytotoxicity from redox cycling anti-cancer drugs results, at least in part, from drug interactions with hydrogen atoms which potentiate the redox cycling pathway(s) ultimately to produce reactive radical species such as the HO$^-$ radicals.

We tested this hypothesis, in this investigation, through a series of time course studies, monitoring the production levels of HO$^-$ radicals from drug solutions during 1 and

![Figure 3: Generation of OH$^-$ radicals from ultrasound and ultrasound + adriamycin treatments as determined via Bz fluorescence at 37°C. [ADR] = 5 μM, [Bz] = 700 μM, ultrasound frequency = 1 MHz, intensity = 1 W cm$^{-2}$]
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were stored at 5°C. All drug aliquots were allowed to warm to room temperature (21°C) and dissolved in sterile PBS + Bz to the final working concentration of 5 μM.

The schematic diagram in Figure 1 illustrates the ultrasound exposure set-up. For consistency in reporting and analysing the data at the two different frequencies, the cylindrical sample sonication chamber was positioned at the characteristic axial distance from the transducer front face at which the near field zone ends and the far field zone begins, viz. at the point where the ultrasound intensity reaches the last maximum value and thereafter decreases inversely with the radial distance from the transducer. The two end faces of the sample chamber were acoustically transparent with 0.002 in thick Mylar windows. Maximum drug solution capacity of the sample chamber was 30 cm³. The water-bath temperature was controlled with an immersion heater to ±0.1°C. The average in vitro acoustic intensity (spatial average and temporal average) was determined by the measured acoustic power by a radiometry technique (without allowing beam spreading), divided by the surface area of the transducer’s aperture.

With continuous 1 W cm⁻² ultrasound exposure, volumes of 1 cm³ of drug + Bz solution were drawn out

Materials and methods

Phosphate buffer solution (PBS) was employed as the working medium in this study. PBS was freshly made in double deionized water (ddH₂O) for each set of experimental conditions. Benzoic acid (Bz) was employed as the OH⁺ reporter molecule. Bz is initially non-fluorescent and upon HO⁺ attack turns permanently fluorescent around 407 nm when excited at 305 nm. Each experimental run contained [Bz] at 100 μg cm⁻³ (700 μM) in PBS prior to ultrasound treatments. Adriamycin (ADR), mitomycin C (Mito C), 5-fluorouracil (5-Fu) and methotrexate (MTX) were purchased from Sigma Chemicals and were dissolved in sterile (0.9% NaCl) saline solution. All drug aliquots were made in sterile PBS and stored in the dark until required. Aliquots of ADR were stored at -20°C and all other drug aliquots

3 MHz ultrasound exposures at an intensity level of 1 W cm⁻². Free radical scavengers and a transition metal chelating agent were employed independently to shunt the HO⁺ generating pathway in order to elucidate the different chemical species involved within the HO⁺-producing pathway(s).

Figure 4: Generation of OH⁺ radicals from ultrasound and ultrasound + mitomycin C treatments as determined via Bz fluorescence at 37°C. [MitoC] = 5 μM, [Bz] = 700 μM, ultrasound frequency = 1 MHz, intensity = 1 W cm⁻²

Figure 5: Generation of OH⁺ radicals from ultrasound and ultrasound + 5-fluorouracil treatments as determined via Bz fluorescence at 37°C. [5-Fu] = 5 μM, [Bz] = 700 μM, ultrasound frequency = 1 MHz, intensity = 1 W cm⁻²
Figure 6 Generation of OH\(^{+}\) radicals from ultrasound and ultrasound + methotrexate treatments as determined via Bz fluorescence at 37°C. [MTX]=5 μM, [Bz]= 700 μM, ultrasound frequency =1 MHz, intensity=1 W cm\(^{-2}\).
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Discussion

The findings from this investigation support the hypothesis that acoustic cavitation plays an active role in enhancing the HO' production from the two clinical anti-cancer drugs ADR and Mito C, which have been previously shown to redox cycle\textsuperscript{16}. In contrast, the non-redox cycling clinical drugs 5-Fu and MTX did not exhibit any significant enhancement in the HO' production. Quinone-containing anti-cancer drugs have been observed to redox cycle in the presence of enzymatic reductase systems which donate electrons to the quinone moiety of the drug molecule and thereafter are autoxidized in the presence of molecular oxygen (Figure 11). The superoxide anion could dismutate into H$_2$O$_2$ or chemically reduce a wide range of substrates\textsuperscript{17}, including the biologically relevant transition metals Fe, Cu and Mg. The potentiation of the Fenton pathway in the presence of H$_2$O$_2$ is well known to generate OH' radicals.

Acoustic cavitation is known to be a principal non-thermal mechanism which potentiates and catalyses chemical reactions\textsuperscript{11}. In accordance with the theory of acoustically induced cavitation, an 'appropriate' intensity ultrasound wave propagating in an aqueous medium can

with the sonicated drug-free treatments (Figure 5). Interestingly, the non-redox cycling drug MTX revealed a marked reduction in the production of HO' radicals relative to the sonicated drug-free media (Figure 6), possibly because MTX can scavenge the radicals.

Ultrasound treatments for 30 min on the four clinical anti-cancer drugs did not yield measurable changes in their chemical constitution, as assessed through HPLC. The chromatographs obtained before ultrasound exposure, and 30 min after the ultrasound exposures, with all drug solutions investigated, were identical, and for this reason the data are not shown.

Free radical scavenger studies performed in the ADR solution at 1 MHz revealed (a) the ability of mannitol to compete actively with the Bz for the HO' radicals (Figure 7); (b) the ability of super oxide dismutase (SOD) to decrease dramatically the HO' yield, thus implicating an active role for O$_2^-$ in producing HO' (Figure 8); (c) the ability of catalase to decrease dramatically the HO' level, which implies an active role of H$_2$O$_2$ in the production of HO' (Figure 9); and (d) the ability of EDTA to decrease significantly the HO' yields, which strongly suggests an active role of transition metal cations in the production of HO' (Figure 10).

Figure 7 Influence of mannitol on the generation of OH' radicals from ultrasound and ultrasound + adriamycin treatments as determined via Bz fluorescence at 37°C. [Mann.] = 700 µM, [ADR] = 5 µM, [Bz] = 700 µM, ultrasound frequency = 1 MHz, intensity = 1 W cm$^{-2}$

Figure 8 Influence of superoxide dismutase on the generation of OH' radicals from ultrasound and ultrasound + adriamycin treatments as determined via Bz fluorescence at 37°C. [SOD] = 23.3 µg cm$^{-3}$ = 100 U cm$^{-3}$, [ADR] = 5 µM, [Bz] = 700 µM, ultrasound frequency = 1 MHz, intensity = 1 W cm$^{-2}$
hydroxylation and is converted into salicylic acid (as independently confirmed by HPLC in this investigation) and thereafter yielded stable fluorescence. The fluorescence assay of salicylic acid, in the present work, allowed the detection of HO\(^{\cdot}\) to 1 nM resolution.

Theoretical determinants of acoustic cavitation thresholds are difficult owing to many parameters of the acoustic field and of the thermodynamic state of the media. However, over the course of numerous

induce stable and/or transient bubble activities. The dynamic response of the bubbles to the ultrasonic wave is extremely rapid (typically ca 1/f) and in the compression phase of the pressure cycle there is insufficient time for thermal diffusion to occur from the interior of the cavities to their local surroundings. Under this condition, adiabatic thermodynamics are applicable and theoretical calculations for transient cavitation have estimated the final temperature of the enclosed water vapour to reach several thousand kelvin with attending pressures of several hundred atmospheres\(^{18-20}\). Hydrogen atoms and HO\(^{\cdot}\) radicals are produced due to complete thermal dissociation of the water vapour. The production of hydrogen atoms from acoustic cavitation has been detected previously with electron spin resonance techniques\(^{12,13}\) and is strongly implicated in this work to have potentiated the two quinone-containing anti-cancer drug redox cycling pathways. The hydrogen atoms and HO\(^{\cdot}\) radicals have been observed to recombine into H\(_2\) or H\(_2\)O\(_2\). The production of HO\(^{\cdot}\) from acoustic cavitation in drug-free media has been previously detected with benzoic acid\(^{14}\). Benzoic acid is initially non-fluorescent and upon OH\(^{\cdot}\) attack undergoes aromatic

**Figure 9** Influence of catalase on the generation of OH\(^{\cdot}\) radicals from ultrasound and ultrasound + adriamyycin treatments as determined via Bz fluorescence at 37°C. [Catalase] = 333 \(\mu\)g cm\(^{-3}\) = 6667 U cm\(^{-3}\), [ADR] = 5 \(\mu\)M, [Bz] = 700 \(\mu\)M, ultrasound frequency = 1 MHz, intensity = 1 W cm\(^{-2}\)

**Figure 10** Influence of EDTA on the generation of OH\(^{\cdot}\) radicals from ultrasound and ultrasound + adriamyycin treatments as determined via Bz fluorescence at 37°C. [EDTA] = 10 mM, [ADR] = 5 \(\mu\)M, [Bz] = 700 \(\mu\)M, ultrasound frequency = 1 MHz, intensity = 1 W cm\(^{-2}\)

**Figure 11** A representative redox cycling pathway of ADR
experiments and detections of cavitation products, a
general trend has emerged from empirical observations,
namely that the 'probability' of inducing acoustic
cavitation is found to be inversely proportional to the
square root of the ultrasound frequency when the acoustic
intensity is held constant. From these empirical
observations it is known that 1 MHz ultrasound of
intensity 1 W cm⁻² is above the cavitation threshold,
whereas 3 MHz ultrasound at 1 W cm⁻² is below the
cavitation threshold, hence it is not surprising to find
a complete lack of OH⁻ production at the higher
3 MHz frequency.

Free radical scavenger findings from 1 MHz ultrasound
in the presence and absence of ADR strongly implicate
an active role of O₂⁻ and H₂O₂ in the production of
OH⁻ radicals. Mannitol is a known HO⁻ scavenger
and was independently employed to compete with benzoic
acid for the available HO⁻ radicals. SOD was employed
independently to dismutase rapidly any formed O₂⁻ into
H₂O₂ in order to prevent chemical reduction of any
natural contaminations of transition metals from the PBS
reagents, which could initiate the Fenton's pathway. Catalase
was employed independently to disassociate rapidly any formed H₂O₂, thereby removing this required
substrate in the activation of the Fenton pathway. A
substantial concentration of EDTA was required to
sequester iron(III) and partially to inhibit it from
participating in the Fenton pathway. Hence the
significant inhibitory effect on HO⁻ production in this
study strongly suggests that the enhancement in HO⁻
production is likely via the Fenton pathway.

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References

1 Yumita, N., Okumura, A., Nishigaki, R., Umemura, K.,
and Umemura, S. The combination treatment of ultrasound and
antitumor drug on Yoshida sarcoma Jpn J Hyp Oncol (1987) 3
175-182.
2 Loverock, P., ter Haar, G., Ormerod, M.G. and Imrie, P.R. The
effect of ultrasound on the cytotoxicity of adriamycin Br J Radiol
(1990) 63 542-546.
3 Harrison, G.H., Balcer-Kubiczek, E.K. and Eddy, H.A. Potentiation
59 1453-1466.
4 Saad, A.H. and Hahn, G.M. Ultrasound enhanced toxicity on
Chinese hamster ovary cells in vitro Cancer Res (1989) 49
5931-5934.
5 Akimoto, R. An experimental study on enhancement of the effect
of anti-cancer drug by ultrasound Jpn Soc Cancer Ther (1985)
20 562-570.
6 Sinha, B.K. and Mimnaugh, E.G. Free radicals and antitumor
drug resistance: oxygen free radicals in the mechanisms of drug
cytotoxicity and resistance by certain tumors Free Rad Biol Med
(1990) 8 567-581.
7 Keizer, H.G., Pinedo, H.M., Schuurhuis, G.J. and Joenje, H.
Doxorubicin (adriamycin) a critical review of free radical-
dependent mechanisms of cytotoxicity Pharmacol Ther (1990) 47
219-231.
8 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 Rad Biol
9 Rino, A. and Mitchell, J.B. Potentiation and protection of
doxorubicin cytotoxicity by cellular glutathione modulation
10 Lele, P.P. Advanced ultrasonic techniques for local tumor
11 Suslick, K.S. Homogeneous sonochemistry. In Ultrasound Its
Chemical, Physical, and Biological Effects (Ed Suslick, K.S.) VCH,
12 Makino, K., Mossoba, M.M. and Riesz, P. Chemical effects of
ultrasound in aqueous solutions. Evidence for "OH and "H by
13 Makino, K., Mossoba, M.M. and Riesz, P. Chemical effects of
ultrasound in aqueous solutions. Formation of hydroxyl radicals
14 Parke, A.V.M. and Taylor, D. The chemical action of ultrasonic
15 Rooney, J.A. Determination of acoustic power outputs in
microwatt-milliwatt range Ultrason Med Biol (1973) 1 13-16.
16 Kalyanaraman, B., Perez-Reyes, E and Mason, R.P. Spin-trapping
and direct electron spin resonance investigations of the redox
metabolism of quinone anti-cancer drugs Biochim Biophys Acta
17 Halliwell, B and Gutteridge, J.M.C. (Eds) Free Radicals in Biology
18 Flint, E.B. and Suslick, K.S. The temperature of cavitation Science
19 Noltingk, B.E. and Neppiras, E.A. Cavitation produced by
20 Neppiras, E.A. and Noltingk, B.E. Cavitation produced by
ultrasound: theoretical conditions for the onset of cavitation Proc
21 Apfel, R.E. and Holland, C.K. Gauging the likelihood of cavitation
from short pulse, low duty cycle diagnostic ultrasound Ultrason
22 Graf, E., Mahoney, J.R., Bryant, R.G. and Eaton, J.W. Iron-
3620-3624.