In vitro interaction of lithotripter shock waves and cytotoxic drugs

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Summary  The effect of a combination of lithotripter shock waves and cytotoxic drugs was examined in vitro. L1210 cells in suspension were exposed to shock waves during incubation with cisplatin, doxorubicin, daunorubicin, THP-doxorubicin, or aclacinomycin. Proliferation was determined using the 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide assay. Dose enhancement ratios were calculated for each drug in order to determine the effect of the additional exposure to shock waves. In addition, partition coefficients and IC₅₀ of the drugs were determined. It was found, that the dose enhancement ratios increased for the drugs with decreasing cytotoxicity. The effect of all five drugs was enhanced by shock waves to a higher degree at 7 min incubation as compared to 50 min incubation. The effect of cisplatin was most significantly enhanced, with a dose enhancement ratio of 6.7 at 7 min incubation. The enhancement increased with the operating voltage used for generating the shock waves, and was only present when cells were exposed to shock waves during the incubation with the drug. An increase in cellular membrane permeability is proposed as the mechanism of interaction between shock waves and drugs.

Lithotripter shock waves are pressure pulses of high amplitude and short duration which are used in medicine for the disintegration of urinary and biliary calculi (Chaussy et al., 1980; Sauerbruch et al., 1986). In liver and kidney they can cause haemorrhages, vessel wall damage and venous thrombi (Delius et al., 1988; Jaeger et al., 1988; Ponchon et al., 1989). The increasing knowledge of the side effects of shock waves in tissues led to investigations of their effect on tumour tissue. Several authors found reduction of tumour volume or even complete remission in small experimental tumours (Russo et al., 1986; Oosterhof et al., 1990; Weiss et al., 1990). In some experiments, the effect could be enhanced when cytotoxic drugs were given in combination with shock waves (Randazzo et al., 1988; Holmes et al., 1990; Lee et al., 1990; Oosterhof et al., 1990; Weiss et al., manuscript in preparation). An enhanced effect of a combined application of shock waves and cytotoxic drugs was also demonstrated in tumour cell cultures (Oosterhof et al., 1989; Wilmer et al., 1989; Lee et al., 1990). Recently, it was reported that ultrasound can enhance the cytotoxicity of doxorubicin (Loverock et al., 1990).

Only a limited number of cytotoxic drugs has so far been tested in combination with shock waves. In this study the combined effect of shock waves and five cytotoxic drugs, cisplatin, and the anthrapylicine antibiotics doxorubicin, daunorubicin, THP-doxorubicin, and aclacinomycin, was investigated in vitro. Substantial differences were noted concerning the enhancement by the combined treatment, raising the question as to the relevant physical or chemical properties of the substances on the one hand and the mechanism of this interaction of shock waves and drugs on the other hand. It was hypothesised that lipophilicity, as one determinant of drug uptake, and cytotoxicity might be properties influencing the interaction of shock waves and drugs.

Materials and methods

Cell line

L1210 mouse leukaemia cells (kindly provided by Dr. H.P. Kraemer, Behringwerke, Marburg, Germany) were grown at 37°C as suspension culture in Nunclon®-flasks (Nunc, Wiesbaden, Germany) in RPMI 1640 medium containing 15% heat inactivated foetal calf serum, 2% sodium pyruvate, and 1% antibiotic-antimycotic solution (Gibco, Eggenstein, Germany) in a humidified atmosphere containing 5% CO₂. Under these conditions, cells proliferated with a doubling time of 11–12 h. In all experiments, log phase single cell suspensions, harvested without trypsinisation, and with a viability greater than 98% were used.

Shock waves and exposure vials

The principle of electrohydraulic shock wave generation has been described earlier (Forssmann et al., 1977). Briefly, shock waves were generated with a Dornier XL1 lithotripter (Dornier Medizintechnik, Gunzenberg, Germany) by underwater spark discharge between the two tips of an electrode located in a metal hemi-ellipsoid which was used as focusing device. The generator was operated at 80 nF capacitance, and a voltage of 25 kV, unless otherwise indicated. The discharge rate was 100 min⁻¹. For the experiments electrodes were not used prior to the first 100 discharges. As one electrode was used per vial, the electrode condition was similar in all experiments. The water in the lithotripter tank, maintained at 35–37°C, was degassed by a vacuum pump; oxygen content, as determined with an oximeter OXI 96 (WTW, Weilheim, Germany), was 0.5–1 mg l⁻¹. According to pressure measurements (Mueller, 1990) shock waves generated at operating voltages of 20 kV and 26 kV have peak positive pressures of 82 MPa and 92 MPa respectively; the focal regions, defined as the isobar representing 50% of peak positive pressure, are 22 mm–5 mm and 31 mm–8.8 mm respectively (length-width).

The cells were exposed in polypropylene vials with an inner diameter of 11.7 mm and a height of 47 mm (Intechem, Muenchen, Germany). The vials were positioned so that the geometric focus of the ellipsoid, indicated by the point of intersection of two laser beams, was located 10 mm above their bottom.

Drugs

Cisplatin and doxorubicin were chosen as these compounds are widely used in cancer therapy. Daunorubicin, THP-doxorubicin and aclacinomycin were chosen since these drugs cover a wide range of lipophilicity (Hoffmann et al., 1990). Cisplatin (Behringwerke, Marburg, Germany) was diluted in culture medium before each experiment. Stock solutions of doxorubicin and daunorubicin (Farmitalia, Freiburg, Germany), THP-doxorubicin and aclacinomycin (kindly supplied by Dr H.P. Kraemer, Behringwerke) with 500 μg drug ml⁻¹ were prepared in sterile 0.9% NaCl solution and kept frozen.

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at - 80°C. The drugs were diluted in culture medium before use.

**Viability and proliferation assay**

Viability was determined by trypan blue dye exclusion (Tennant, 1964). Equal amounts of cell suspension and trypan blue (2 mg ml⁻¹ in 0.9% NaCl solution; Fluka, Buchs, Switzerland) were mixed. After 15 min at room temperature the unstained cells were counted in a hemocytometer. Their number in treated samples was calculated as fraction of viable cells from the untreated control in the respective experiment.

Proliferation of cells that were viable after exposure to shock waves and/or drugs was tested with the 3-4,5-dimethy1-2-(3-hydroxy-5-nitrobenzene)- 1,3-diazolo[4,5-c]pyrimidin (MTT) assay (Mosmann, 1983; Carmichael et al., 1987; Twerynowski & Luscombe, 1987). One hundred µl cell suspension, containing 9 × 10⁴ viable cells, and 50 µl culture medium were plated into each well of 96-well round bottom microtitre plates (Nunc, Wiesbaden, Germany) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. At least 12 replicate wells were used to determine each data point. After 72 h, providing time for at least six cell duplications of untreated control cells, 50 µl MTT solution (2.5 mg ml⁻¹ in 0.9% NaCl solution; Sigma, Taufkirchen, Germany) was added to each well. After a further incubation of 4 h the supernatant fluid was removed, 100 µl DMSO (E. Merck, Darmstadt, Germany) was added to each well, and absorbance at 492 nm was measured within 5 min using a 400 AT plate reader (SLT Lab instruments, Overath, Germany). The proliferation of treated samples was calculated as fraction of the proliferation of untreated control cells in the respective experiment.

**Partition coefficient**

Partitioning of the drugs between aqueous and lipid phase was determined by measuring the optical density (OD) of drug solutions (cisplatin at 500 µg ml⁻¹ in 0.9% NaCl solution; doxorubicin, daunorubicin, THP-doxorubicin, and aclacinomycin at 10 µM in 10 mM Tris-HCl, pH 7.0) before and after extraction with an equal volume of n-octanol (Zenerbergh et al., 1982). Measurements were performed at 300 nm for cisplatin, at 435 nm for aclacinomycin, and at 480 nm for daunorubicin, doxorubicin, and THP-doxorubicin. The partition coefficient was calculated according to:

\[ \text{OD}_{\text{after extraction}} / \text{OD}_{\text{before extraction}} - \text{OD}_{\text{after extraction}} / \text{OD}_{\text{before extraction}} \]

**Drug cytotoxicity**

Cytotoxicity of each of the five drugs without shock waves was determined by establishing dose-response curves for a 72 h continuous exposure. Proliferation was determined with the MTT assay with the culture medium containing the appropriate drug. At least three concentrations per drug were tested and the experiment was repeated at least twice for each drug. From the dose-response curves the drug concentrations that inhibited proliferation of L1210 cells by 50% (IC₅₀) were calculated.

**Experimental procedures**

**Simultaneous exposure to shock waves and different drug concentrations**

For the five drugs, dose-response curves were obtained with incubation times of 50 and 7 min, with or without simultaneous exposure to 300 shock waves. Drug concentrations were chosen according to the detectability of reduced proliferation with the MTT assay. At least three concentrations were tested per drug. During the 50 min incubation with the drugs the time of shock wave application was chosen randomly. Previous experiments had revealed no difference of the cytotoxic effect on cells between application of shock waves at the beginning or at the end of a 50 min incubation with cisplatin (Wilmer et al., 1989). Seven minutes was the shortest incubation time that could be tested with this experimental setup. Two or more samples of 5.2 ml cell suspension, containing 2 × 10⁶ viable L1210 cells and the appropriate drug at various concentrations, and two samples with no drug were transferred into the exposure vials. One of these latter samples received no shock wave treatment and served as control, the other was used to assess the effect of exposure to shock waves alone. The vials (not being exposed to shock waves) were placed peripherally in the waterbath outside of the shock wave field. The experiment was repeated at least twice for each drug concentration.

In this and the following experiments, cells were washed twice in a 4-fold volume of Hank’s balanced salt solution immediately after drug and/or shock wave exposure and resuspended in 2 ml culture medium. The number of viable cells was determined and proliferation was assessed.

**Shock waves generated at different operating voltages**

A dose-response curve was obtained with 500 shock waves generated at 15, 20, or 25 kV, with or without simultaneous incubation with cisplatin (16.7 µM) for 50 min. Each experiment consisted of two or more samples of 5.2 ml cell suspension, containing 2 × 10⁶ viable L1210 cells, and one sample of drug without shock wave exposure, serving as control. The experiment was repeated twice for each operating voltage.

**Sequential exposure to shock waves and cisplatin**

Sequential exposure was tested with cisplatin (25 µM). In one series, 500 shock waves at 25 kV were applied before a 7 min incubation with cisplatin (25 gM). In the other series of experiments, shock wave exposure and cisplatin incubation was 3 waves each, the shortest interval that could be tested with this setup. In another series, 7 min incubation with cisplatin was done first. Cells were washed twice in a 4-fold volume of cold Hank’s balanced salt solution and resuspended in fresh culture medium. Due to this, the interval between the end of cisplatin incubation and exposure to 500 consecutive shock waves at 25 kV was 60 min. The experiments were repeated twice for each exposure sequence.

**Data analysis**

Relative proliferation is given as mean values ± standard deviation of at least three independent experiments. In the experiments with simultaneous exposure to shock waves and drugs, treatment with shock waves alone reduced the relative proliferation of viable cells to 0.86 ± 0.10 (n = 49). For the evaluation of the interaction of shock waves and drugs, the proliferation of cells that were additionally exposed to shock waves was normalised for the effect of shock waves alone. Survival curves were fitted to the data by non-linear regression analysis. The ratio of the drug concentration needed to reduce proliferation of L1210 cells by 50% without shock wave exposure divided by the dose needed to reduce proliferation by 50% with shock wave treatment was calculated; the ratios for a proliferation reduced by 60%, 70%, 80% and 90% were calculated in an analogous manner. The dose-enhancement ratio (DER) is given as the mean of these five ratios. Dependency of relative proliferation of cells exposed to cisplatin and shock waves upon the operating voltage and correlation of DER with molecular weight, partition coefficient, and IC₅₀ of the drugs were tested with least-squares linear regression analysis.

**Results**

The dose-response curves for a 50 min incubation with cisplatin, doxorubicin, daunorubicin, THP-doxorubicin, and aclacinomycin with or without additional exposure to 500 shock waves generated at 25 kV are shown in Figure 1. Proliferation was only assessed for cells that had been trypan blue negative after the respective treatment. The curve resulting from exposure to cisplatin alone showed a marked initial
The effect of sequential exposure to cisplatin (25 μM) for 7 min and 500 shock waves at 25 kV before or after cisplatin exposure is shown in Table II. No enhanced effect of the combined treatment could be demonstrated when the cells were exposed to cisplatin before or after shock wave treatment.

Molecular weights, partition coefficients, IC₅₀ values, and DERs resulting from exposure to shock waves during a 50 or 7 min incubation time with the drugs are summarised in Table III. For the highly hydrophilic cisplatin, no partitioning could be detected. With an IC₅₀ of 0.974 μM it revealed the lowest cytotoxicity among the five drugs tested in this

The effect of combined treatment was normalised for the effect of exposure to shock waves alone. Additional exposure to shock waves reduced relative proliferation in a dose-dependent manner (P < 0.05; regression analysis with relative proliferation as dependent variable).

### Table 1

| Treatment                     | Relative proliferation |
|-------------------------------|------------------------|
| Cisplatin                     | 0.70 ± 0.04            |
| Cisplatin + shock waves at 15 kV | 0.47 ± 0.06            |
| Cisplatin + shock waves at 20 kV | 0.25 ± 0.08            |
| Cisplatin + shock waves at 25 kV | 0.17 ± 0.04            |

*Mean values ± s.d.; n = 3. Proliferation after combined treatment was normalised for the effect of exposure to shock waves alone.

### Figure 1

Dose-response curves for L1210 cells incubated for 50 min with cisplatin, daunorubicin, doxorubicin, THP-doxorubicin, or aclacinomycin. Cells were exposed to the drugs alone (O and dashed curves) or additionally treated with 500 shock waves at 25 kV (● and solid curves). Proliferation after combined treatment was normalised for the effect of exposure to shock waves alone. The points and bars represent mean values and standard deviations in three to six independent experiments. For clarity some of the points are slightly offset. Curves were fitted to the data by non-linear regression analysis.

### Figure 2

Dose-response curves for L1210 cells incubated for 7 min with cisplatin, daunorubicin, doxorubicin, THP-doxorubicin, or aclacinomycin. Cells were exposed to the drugs alone (O and dashed curves) or additionally treated with 500 shock waves at 25 kV (● and solid curves). Proliferation after combined treatment was normalised for the effect of exposure to shock waves alone. The points and bars represent mean values and standard deviations in three to six independent experiments. For clarity some of the points are slightly offset. Curves were fitted to the data by non-linear regression analysis.
study. THP-doxorubicin, with a medium partition coefficient of 41.2, showed the highest cytotoxicity with an IC50 of 0.003 μM. DERs as a function of cytotoxicity are shown in Figure 3. The enhanced effect of the combined treatment decreased with increasing cytotoxicity of the drugs. The DERs did not correlate with the molecular weights or the partition coefficients of the drugs.

Discussion

It has previously been demonstrated that shock waves disrupt tumour cells in vitro (Russo et al., 1986; Brümmern et al., 1989; Wilmer et al., 1989; Gambihler et al., 1990). Additionally, shock waves have been reported to enhance the antiproliferative effect of vinblastin (Oosterhof et al., 1989) and cisplatin (Wilmer et al., 1989; Lee et al., 1990). Further examination of a combination of shock waves with anticancer drugs appeared therefore promising.

Substantial differences were not noted between the DERs for the various drugs. Alone the combination of shock waves with cisplatin showed a clear and pronounced effect on cell proliferation. Shock waves combined with THP-doxorubicin or daunorubicin, on the other hand, yielded no enhancement of the effect. Platinum drugs passively diffuse into cells at a very slow rate (Richon et al., 1987). The uptake of THP-doxorubicin is a very rapid process, and its high cytotoxic activity as compared to other anthracyclines was related to the ease with which THP-doxorubicin accumulates in cells (Tapiero et al., 1986). Only small DERs were found for the combination of shock waves with doxorubicin and aclacinomycin. Differences in the rates of uptake between these substances have been reported (Zenebergh et al., 1982), but it appears that the uptake rate, if it is relevant for the combination with shock waves, is important only at very slow rates similar to those of cisplatin. This view is supported by the finding that only for cisplatin the DER was clearly higher with a 7 min incubation as compared to the 50 min incubation while it was similar for the other drugs.

Several mechanisms of the interaction of shock waves and anticancer drugs have to be taken into account. Shock waves could cause ultrastructural changes within the cell making it more susceptible to cytotoxic drugs. L1210 cells exposed to shock waves have been shown to exhibit intracellular alterations (Russo et al., 1987; Brümmern et al., 1989); however, the subpopulation showing these alterations may be identical with cells detected as geometrically intact but nonviable (Brümmern et al., 1989). Furthermore, exposure to shock waves alone only slightly decreased cell proliferation, corresponding to previous results (Brümmern et al., 1989; Wilmer et al., 1989; Gambihler et al., 1990). Finally, DERs from combined exposure to shock waves and anticancer drugs were markedly different for the various substances making an unspecific effect on cells that are considered viable after exposure to shock waves less likely.

Sequential exposure to shock waves and drug did not enhance the cytotoxic effect of cisplatin, thus indicating a short-lived shock wave effect. Such an effect might be mediated by free radicals. Cavitational, which is the generation and movement of bubbles in a fluid (Apfel, 1982; Crum, 1982), is produced by shock waves in the lithotripter waterbath (Coleman et al., 1987). High local temperatures caused by cavitation lead to the formation of free radicals (Makino et al., 1982). Yet, findings about cavitation in intact cells are conflicting, and experiments on supersaturation with gases demonstrated that bubbles were not generated within cells (Hemmingsen & Hemmingsen, 1979). Although the formation of free radicals during shock wave application has been described, cell killing by shock waves did not correlate with their formation (Morgan et al., 1988; Gambihler, submitted). So far, there is no evidence for a major contribution of the high local temperatures or free radicals to the effect of shock waves on tumour cells.

Another possibility of shock wave action is a temporary increase of the permeability of the cellular membrane similar to electrone permeabilisation (Melvik et al., 1986). As the cytotoxic effect of cisplatin was only enhanced when cells were treated with shock waves during drug exposure the increased permeability would be a short-lived effect. An increased membrane permeability by shock waves could also explain, why only the effect of cisplatin was clearly enhanced. Due to its hydrophilic property and the slow intracellular accumulation, it could profit most from an increase in cell membrane permeability. THP-doxorubicin on the other hand, would profit least from an increased membrane permeability. Because of its lipophilic nature, it is rapidly taken up even without shock waves, and exerts cytotoxic activity on L1210 cells similar to that of cisplatin already at a 300 times lower concentration. Thus, a temporary increase in membrane permeability appears to be the most likely explanation for the highly selective effect of shock waves in the enhancement of drug effects. Such a mechanism also allows to explain the dependency of the DERs upon the incubation time. A study on the effect of ultrasound on the cytotoxicity of doxorubicinc has already demonstrated an increased intracellular drug level after combined treatment (Loverock et al., 1990).
INTERACTION OF LITHOTRIPTER SHOCK WAVES AND CYTOTOXIC DRUGS

Our further studies are aimed at the direct determination of intracellular drug concentration after exposure to shock waves. It could be demonstrated that shock waves can cause accumulation of propidium iodide, a fluorescent dye which is normally excluded by an intact cell membrane, in cells that are still able to metabolise fluorescein diacetate, indicating viability. Additional cell sorting experiments revealed that these cells were still able to proliferate (Gambhirb, submitted).

The number of drugs tested in this study has been limited, and further experiments are necessary to determine whether there is a general relation between hydrophilic properties of the drugs, slow uptake and high IC50 on the one hand, and a pronounced enhancement of the drug effect by shock waves on the other hand. Even higher DERs than in this study might be detected with anticancer drugs that by themselves cannot pass the tumour cell membrane. Since shock waves can be well focused even deep in the body, their combination with such drugs would open up the possibility to implement local action of the drug in vivo.

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