Evaluation of suitable solvents for testing the anti-proliferative activity of triclosan - a hydrophobic drug in cell culture

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Triclosan, a broad spectrum antibiotic is currently being evaluated for its anti-cancer property. Though several solvents are available to dissolve lipophilic (hydrophobic) drugs, solubility and toxicity aspects pose a challenge, when combined with the cell culture medium. In this paper, we present a simple approach based on physico-chemical and biologic criteria to choose a suitable solubilizing agent to study the anti-proliferative property of triclosan in breast cancer cell line MCF-7. Triclosan was dissolved in five different solvents viz. DMSO, absolute ethanol, 1 N NaOH, 55% polyethylene glycol + 45% ethanol mixture (PEM) and acetone and diluted with the culture medium (1 mg/ml). Although triclosan dissolved completely in all five solvents, on dilution with culture medium, turbidity was observed in DMSO, 1 N NaOH and ethanol. Cell viability was 95.23% in 10 \(\mu\)l of acetone, when compared with 49.45% at the same volume of PEM. This non-toxic nature of acetone was supported by DNA fragmentation analysis and phase contrast microscopy. A significant decrease in cancer cell proliferation at 100 \(\mu\)g/ml of acetone-solubilized triclosan, compared with 100 \(\mu\)g/ml of PEM-solubilized triclosan (p<0.05) indicated stronger anti-proliferative effect and greater drug-sensitivity of triclosan when solubilized in acetone. Results showed that acetone-solubilized triclosan was suitable for anti-cancer investigations in cultured MCF-7 cells.

Keywords: Triclosan, Cytotoxicity, Cell culture, Drug sensitivity, Acetone, Polyethylene glycol

Triclosan, a broad spectrum antimicrobial agent has also been widely used for the last three decades in soaps, deodorants, cosmetics and toothpastes\textsuperscript{1,2}. It acts as antibacterial agent by inhibiting the enoyl reductase activity of the type II fatty acid synthase in bacteria. Zuckerbraun et al.\textsuperscript{3} studied the \textit{in vitro} cytotoxicology of triclosan, the active ingredient in some mouth rinses and dentifrices used in the prevention and treatment of gingivitis and plaque using the \textit{in vitro} cell culture system of Smulow-Glickman (S-G) human gingival epithelial cell line. Triclosan is also being investigated for its anti-cancer properties due to its inhibition of type I fatty acid synthase, a potential chemotherapeutic target for cancer\textsuperscript{4}.

Since triclosan is insoluble in water, it needs to be solubilized in organic solvents or other formulations to enable its bioavailability. However, its biological activities such as anti-plaque and anti-inflammatory effects are lost, when unsuitable solubilizers are used\textsuperscript{5,6}. Kjaerheim et al\textsuperscript{5} experimented the dissolution of triclosan in different oils (olive, soy, and sunflower oils), as well as in polyethylene glycol (PEG) and glycerol, wherein they found that triclosan dissolved in oils lost its anti-bacterial activity \textit{in vitro}, though the oils in themselves exhibit significant plaque inhibition. The results of their study was of significance in the context of toothpastes and mouth rinses that contain flavoring oils and occasionally also glycerol and PEG, wherein these substances are likely to interfere with the clinical effect of triclosan in these products\textsuperscript{7}. In another study\textsuperscript{8}, it was shown that the nature of the solubilizing agent influenced the anti-oral malodour activity of triclosan.

In order to investigate the anti-proliferative effect of triclosan in cancer cells grown \textit{in vitro}, in this study, we have evaluated the role of the solvent in combination with the specific cell culture medium using a breast cancer cell line (MCF 7 cells). Earlier, the solvents such as DMSO, ethanol, alkali, acetone, and 55% polyethylene glycol-400 + 45% ethanol mixture (PEM) have been used for dissolving triclosan\textsuperscript{4,6,9}. However, in the context of cell culture-
based testing of anti-proliferative (cytotoxic) property of triclosan, no systematic evaluation of solvents has been carried out, so far. We present a simple approach to select a suitable solubilizing agent that would enable testing of a lipophilic drug’s anti-proliferative activity in cells grown in vitro.

Materials and Methods

Materials
Triclosan (2, 4, 4’-trichloro-2’-hydroxydiphenyl ether), MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich, USA. Polyethylene glycol 400 (PEG-400) was obtained from Hi Media (Mumbai, India) and other chemicals were purchased from Merck (Mumbai, India).

Cell lines and culture conditions
Breast (mammary) cancer cell line MCF-7 was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM supplemented with 750 µl/dl calf insulin (40 IU/ml) and 10% fetal bovine serum at 37°C in an atmosphere with 5% CO₂.

Test of solubility and pH
Triclosan was dissolved in five different solvents: (i) DMSO, (ii) acetone, (iii) 55% PEG-400 + 45% ethanol mixture (PEM), (iv) absolute ethanol, and (v) 1 N NaOH, and then diluted with the culture medium to obtain a final concentration of 1 mg/ml. The drug was considered completely solubilized when it formed a clear solution in its solvent and produced no turbidity or precipitate, when diluted with the medium. Any precipitation or turbidity observed visually in the final solution containing the drug + solvent + medium was recorded. The pH was measured with a pH meter (LI120-ELICO India).

Cell viability (MTT) assay
MCF-7 cells were seeded at 2.5 × 10³ cells/well in 96-well plates with 100 µl of culture media and incubated at 37°C for 24 h and then fresh medium was added. Triclosan dissolved in acetone and PEM (selected after the above-mentioned preliminary screening of five different solvents) was added at a range of concentrations from 50-200 µg/ml to the wells. The solvent controls contained the corresponding volumes (5-20 µl) of solvent alone, without the drug. The cells were then incubated for 48 h at 37°C. Thereafter, the medium was removed and 100 µl of fresh medium and 10 µl of MTT (5 mg/ml) was added to all the wells and incubated for 4 h at 37°C. The supernatant was then removed and 100 µl DMSO was added to dissolve the formazan crystals. Color developed was read at 570 nm. Cell viability (or cell survival) was calculated as: (Test OD/Control OD) × 100. The 50% inhibitory concentration (IC₅₀) of triclosan solubilized in acetone and in PEM was calculated using polynomial regression analysis using Microsoft Excel.

Assessment of cell morphology by phase contrast microscopy
MCF-7 cells treated with PEM and acetone were assessed for morphological changes using a phase contrast microscope (Nikon). The cells were exposed to these solvents in volumes corresponding to the volume required to dissolve triclosan at its 50% inhibitory concentration IC₅₀.

DNA fragmentation assay (Agarose gel electrophoresis)
MCF-7 cells were plated at 2.5 × 10⁴ cells/well in 12-well plates and incubated for 24 h. Then the medium was removed and fresh medium and respective solvent controls (5-20 µl) were added to the wells and incubated for 48 h. At the end of incubation, the cells were collected and pelleted and the DNA was extracted by Qiagen (Germany) kit as per the manufacturer’s instructions. The extracted DNA was electrophoresed at 100 V in a 2% agarose gel with 0.5% ethidium bromide for 2 h. The obtained DNA fragments were visualized under UV light and documented with a gel documentation system (Vilber Lourmat, France). The DNA from solvent-incubated cells were run along with that of untreated MCF-7 cells (control), toxic H₂O₂-treated cells as positive control for DNA laddering, and a 100 bp marker.

Statistical analysis
The results were expressed as mean ± SD of values obtained from a minimum of three independent experiments, each performed in triplicate. In all the experiments, the untreated cells with the culture medium served as the control. Comparisons between specific groups as mentioned in the results section were done using student’s t-test.

Results
Solubility of triclosan
Differences in triclosan solubility were observed in the five solvents made up with the culture medium.
(final conc: 1 mg/ml) (Fig. 1). Triclosan did not dissolve completely in DMSO (bottle 1), absolute ethanol (bottle 2) and 1 N NaOH (bottle 3) when diluted with culture medium, resulting in a turbid solution. However, PEM (bottle 4), and acetone (bottle 5) solubilized triclosan diluted with the culture medium resulted in a clear solution. pH was also recorded in these triclosan + solvent + culture medium solutions. The pH of the final solutions with 1 N NaOH and absolute ethanol was unfavorable (pH 8.0) for cell culture conditions while with acetone, PEM and DMSO an optimal pH of 7.4 was obtained. Based on the solubility and pH criteria, only two solvents (acetone and PEM) were selected for further evaluation of suitability as triclosan solubilizers in cell culture conditions.

**Effect of solvents on cultured cells**

Toxicity of the solvents (5, 7.5, 10 and 20 µl) was evaluated in terms of percentage viability of MCF-7 cells incubated with the solvents only. Fig. 2 represents MTT assay results of acetone and PEM, respectively. A significant decrease was observed in the viability of cells incubated with PEM, in comparison with those incubated with acetone (p<0.05). At the same volume (10 µl), while acetone-incubated cells had 95.23% viable cells, PEM-incubated cells had only 49.45% viable cells, strongly indicating the cytotoxicity of PEM in MCF-7 cell culture conditions.

Cell morphology analysis (Fig. 3c) showed shrinkage of cells with PEM in MCF-7 cells, while there was no observable change in the morphology of cells incubated with acetone (Fig. 3b). Further, agarose gel electrophoresis revealed mild oligonucleosomal DNA fragmentation (Fig. 4) with PEM, suggesting its cytotoxicity. On the other hand, acetone-incubated group was comparable with the untreated control (Fig. 5).
Effect of solvents on anti-proliferative activity of triclosan

The anti-proliferative effect of triclosan was assessed, when solubilized in acetone and PEM by MTT assay (Fig. 6). Panels of triclosan concentrations between 50-200 µg/ml were chosen. The IC\textsubscript{50} (50% inhibitory concentration of the drug over cell viability) was obtained by interpolation. IC\textsubscript{50} of triclosan solubilized in acetone was found to be 91.09 µg/ml, while in PEM, it was 121.25 µg/ml. This implied that a 1.33-fold increase in drug concentration was required to induce anti-proliferative effects in the cells, when triclosan was dissolved in PEM in comparison with acetone-dissolved triclosan, indicating lesser drug-sensitivity in PEM. A significant decrease in cell proliferation at 100 µg/ml of acetone-solubilized triclosan, compared with 100 µg/ml of PEM-solubilized triclosan (p<0.05) was observed, indicating a stronger anti-proliferative effect and greater drug sensitivity of triclosan when solubilized in acetone.

Discussion

Triclosan has been known for its broad-spectrum anti-microbial activity for nearly three decades\textsuperscript{10} and is also reported to inhibit the mediators of inflammation, thereby exhibiting anti-inflammatory effects\textsuperscript{11}. It is now being investigated for its anti-proliferative activity and seems to have promising anti-cancer effects\textsuperscript{12}. Being a lipophilic organic molecule, solubilizing triclosan in a solvent, followed by culture medium posed some difficulties in testing triclosan’s anti-proliferative activity in cells grown in vitro. Here, we describe a systematic approach for deciding a solvent that not only dissolves the hydrophobic drug, but is also compatible biologically for experimentation with triclosan on cells grown in vitro.

Triclosan is an important anti-microbial constituent in personal care and oral care products. Being sparingly soluble in water, product formulations take into account the solubilizers that are effective in not only producing stable formulations, but also maximize the anti-bacterial efficacy of triclosan in the formulation. In this context, Taylor et al.\textsuperscript{13} suggested formulation approaches, wherein they clearly demonstrated an association between the antimicrobial activity and the physico-chemical characteristics of triclosan in aqueous solution, solution with added co-solvent (propylene glycol) and surfactant solutions (sodium lauryl sulphate and ammonium lauryl sulphate)\textsuperscript{13}. We found that although triclosan was completely soluble in DMSO, NaOH and ethanol, dilution with culture medium ideal for growing MCF-7 cells resulted in turbidity or unfavorable pH change that was not conducive for further testing in cultured cells. On the other hand, both in acetone and PEM a clear solution of solubilized triclosan was obtained in culture medium. However, we further sought to verify amongst these
two solvents, if any of them interfered with the biologic function of triclosan in inhibiting proliferation of MCF-7 cells in vitro.

Our results clearly indicated that acetone-solubilized triclosan was suitable for testing the drug in MCF-7 cells grown in vitro. Acetone was shown to be a non-cytotoxic solvent (an average of 97% cell viability in the solvent in volume ranging from 5-20 µl). This was in contrast with the cytotoxicity of PEM solvent on MCF-7 cells with an average cell viability of 58% in the selected range of solvent volumes (5-20 µl). Acetone incubated MCF-7 cells showed intact DNA, while PEM incubated MCF-7 cells showed DNA fragmentation on agarose gel electrophoresis, suggestive of its toxicity on MCF-7 cells.

The study, particularly in dental research have investigated the role of solvents in determining the biological efficacy of triclosan. In a study by Kjaerheim et al., it has been reported that triclosan, dissolved in a suitable solvent has an antiplaque effect and that the nature of detergent or organic solvent used to dissolve triclosan affects its clinical effect.

The IC_{50} of triclosan in acetone on MCF-7 cells was 91.09 µg/ml, while that of triclosan solubilized in PEM showed a rise by 1.33-fold to 121.25 µg/ml. The higher IC_{50} of triclosan in PEM implied a decreased drug-sensitivity in PEM, which might be due to the large molecular size of PEG-400 known to form micelle-like structures. These may bind with the triclosan molecule, thus rendering it less available for biological activity. The marked reduction in cell viability at 100 µg/ml (p<0.05) on treatment with acetone-solubilized triclosan in comparison with PEM-solubilized triclosan clearly indicated greater biological activity of triclosan dissolved in acetone in countering the proliferation of MCF-7 cells grown in vitro.

Testing water-insoluble drugs in cells grown in vitro often requires organic solvents to solubilize the drug in culture medium that may result in toxicity on the cells and may interfere with the biological activity of the test-drug. Factors, such as the solvent volume required to solubilize the drug, duration of incubation with the cells and the specific chemistry of the solvent and drug (alone and in combination) would determine the choice of solvent. Further, the suitability of solvent with respect to the specific type of cell line needs to be evaluated. For instance, Pace and Elliott found that various cell lines grown in vitro showed differences in their degree of sensitivity to different chemicals such as acetone and phenol. They found that the skin cells are more sensitive to acetone’s effects than the fibroblast cells, wherein a concentration of 5.0 mg/ml is not toxic to fibroblasts within a 10-day period, although it is noticeably toxic to the skin cells.

To conclude, acetone demonstrated favorable physico-chemical and biologic characteristics to solubilize triclosan and to study its anti-proliferative effect in a breast cancer cell line. This step-wise approach of selecting a solubilizing agent can also be extended to other hydrophobic drugs for evaluation of their anti-proliferative potential in cultured cells. The results of this study bring out different aspects of a solvent-cell culture system emphasizing on criteria such as solubility, pH, solvent toxicity, and interference of solvent with drug efficacy. With the advent of newer pharmaceutical solvents, such as room temperature ionic liquids (green solvents) that greatly increase the solubility of water-insoluble drugs, their effects in cell culture system would need careful and systematic examination.

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References
15 Pace DM & Elliot A (1962) *Cancer Res* 22, 107-112