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ORIGINAL ARTICLE

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Povidone-iodine results in rapid killing of thymic epithelial tumour cells through cellular fixation†

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EXPERIMENTAL

Key question

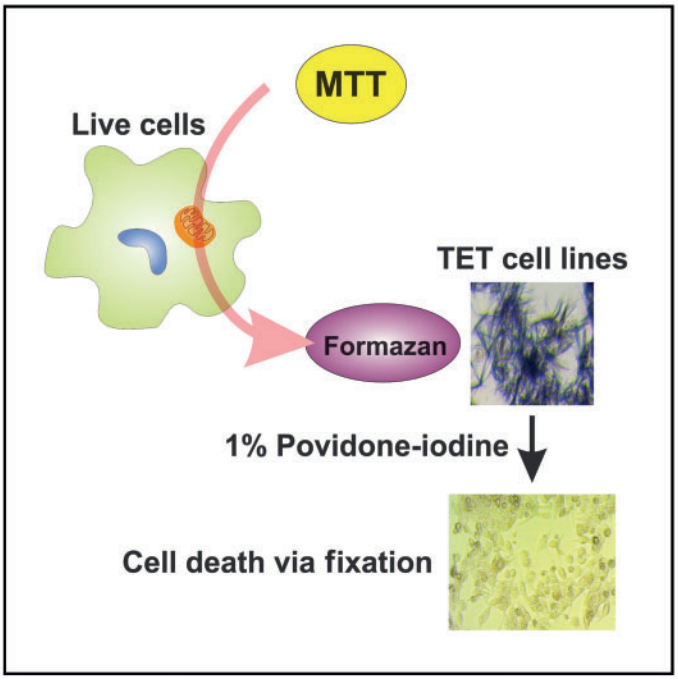
What is the mechanism of cytotoxic effect of povidone-iodine (PVP-I) on thymic epithelial tumour cells?

Key finding(s)

PVP-I results in rapid death of human thymic epithelial tumour (TET) cells and normal mesothelial cells through a cellular fixation mechanism.

Take-home message

PVP-I results may favourably impact control of micrometastatic disease following resection of thymic epithelial tumours with pleural dissemination.



Abstract

OBJECTIVES: Hyperthermic pleural lavage with povidone-iodine (PVP-I) is utilized to control micrometastatic disease following cytoreductive surgery for thymic epithelial tumours (TETs). Our objective was to investigate whether PVP-I demonstrates direct cytotoxicity against human TET cells.

METHODS: Human Met-5A (immortalized mesothelial cell), IU-TAB-1 (thymoma) and Ty-82 (thymic carcinoma) cell lines were treated with serial dilutions of PVP-I (0.01–10%) for 5, 30 and 60 min at 37°C and 42°C. MTT assays and flow cytometry were used to evaluate cell death and apoptosis. Membrane permeability was assayed by intracellular staining of cleaved poly-ADP-ribose polymerase. Cellular fixation was evaluated by membrane disruption of dead cells by dimethylsulphoxide and by comparing cleaved poly-ADP-ribose polymerase staining following PVP-I with known fixatives.

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RESULTS: MTT assays demonstrated that PVP-I concentrations greater than 0.5% led to rapid cell death in both TET cell lines regardless of temperature. IC_{50} values following 5 min of exposure to PVP-I were 8.4 mM (0.3%) and 13.3 mM (0.48%) for IU-TAB-1 and Ty-82, respectively and 8.9 mM (0.32%) for MeT-5A. Flow cytometry demonstrated that 5-min exposure of either cell line to 1% PVP-I resulted in profound cell death: 74% and 58% at 5 min and 97% and 95% at 30 min, for IU-TAB-1 and Ty-82 cells, respectively. Resistance of PVP-I-treated cells to dimethylsulphoxide lysis and similar cleaved poly-ADP-ribose polymerase expression following PVP-I and known fixatives revealed cellular fixation as the mechanism of death following PVP-I exposure.

CONCLUSIONS: PVP-I results in rapid death of human TET cells and normal mesothelial cells through a cellular fixation mechanism and may, therefore, favourably impact the control of micrometastatic disease following resection of TETs with pleural dissemination.

Keywords: Thymic epithelial tumour • Povidone-iodine • Pleural dissemination • Cytotoxicity • Hyperthermic pleural lavage

INTRODUCTION

Thymic epithelial tumours (TETs) including thymomas and thymic carcinomas (TCs) are rare and enigmatic malignancies that have a biological propensity for local invasion and regional dissemination. Surgical resection is the mainstay of treatment for earlier stage TETs (Stages I–III) in which tumour cells are confined to a solitary primary tumour, even though the primary tumour demonstrates invasion of a neighbouring organ (Stage III). Pleural dissemination (Masaoka–Koga Stage IVA [1]) is seen in up to 14% of patients with TETs [2–6] and represents tumour cell escape from the primary tumour and implantation in the pleural space. The clinical presentation of Stage IVA TETs can range from 1 or several small isolated 'drop metastases' on the visceral and/or parietal pleura to a thick sheet of tumour encasing pleural space, forming an invasive rind around the entire lung and potentially involving the chest wall, pericardium and diaphragm.

The treatment of patients experiencing TETs with pleural involvement is commonly centred upon surgical resection. Resection of pleural metastases is often performed in the setting of a multimodal treatment approach that includes chemotherapy and/or radiotherapy, which is thought to extend overall survival (OS) and recurrence-free survival in select patients [4]. The 2 largest cohort studies (the European Society of Thoracic Surgeons (ESTS) Thymic Working Group ($n = 152$) [7] and the Japanese Association for Research on the Thymus (JART; $n = 118$) [8]) of surgically treated TETs with pleural dissemination were recently reported, and although they are subject to the biases of retrospective studies, their outcomes were relatively concordant. Both studies demonstrated favourable long-term surgical outcomes following pleural resection and found complete macroscopic resection to be an independent predictor of improved OS. The ESTS report showed that TET patients (thymoma and TCs) with pleural involvement treated with surgery had a 5-year OS rate of 87% and a 10-year OS rate of 63%, and the JART study demonstrated similar 5-year OS of 87% for thymomas with pleural dissemination.

Resection of Stage IVA TETs involves thymectomy and resection of pleural metastatic disease often by pleurectomy/decortication or extrapleural pneumonectomy, depending on the burden and invasiveness of disease. Although complete 'macroscopic' resection of pleural disease can be accomplished by pleurectomy/decortication or extrapleural pneumonectomy, most patients have residual 'microscopic' disease and, hence, are at a high risk of pleural recurrence. To treat residual microscopic disease (and, therefore, extend the 'surgical margin'), a number of local treatment strategies have been utilized immediately following the removal of the tumour, including photodynamic therapy [9], heated intraoperative chemotherapy lavage [10] and povidone-iodine [11].

Povidone-iodine (PVP-I) is a time-honoured antiseptic agent used for handwashing, skin preparation for surgery and antiseptic irrigation. Recent studies have reported that PVP-I has a cytotoxic property against certain cancer cell lines including malignant pleural mesothelioma (MPM), colorectal cancer, breast carcinoma, lung carcinoma and melanoma [12–15], with a mitochondria-mediated apoptosis pathway proposed as the mechanism of PVP-I-induced cell death [12, 14]. Recent clinical studies have incorporated intrapleural administration of heated 1% or 10% PVP-I into a multimodal treatment approach against MPM [16] and Stage IVA TETs [11] and have demonstrated safety and feasibility. Whether PVP-I is cytotoxic towards TETs cells and whether hyperthermia enhances its antitumour efficacy, are unknown. We, therefore, set out to elucidate the effects of PVP-I, under normothermic and hyperthermic conditions, on human TETs cells.

MATERIALS AND METHODS

Cell lines

Two TET cell lines (IU-TAB-1, a cell line derived from an AB1 thymoma and Ty-82, a TC cell line) and a normal mesothelial cell line (MeT-5A, derived from SV40 transfected and immortalized normal mesothelial cells) as control cells were used in the present study. The IU-TAB-1 cell line was kindly provided by Dr Yesim Gokmen-Polar at Indiana University, and the Ty-82 cell line was purchased from the JCBR cell bank (Sekisui XenoTech, LLC, Kansas City, KS, USA). The MeT-5A cell line was purchased from the ATCC (https://www.atcc.org/). TET cell lines were cultured in roswell park memorial institute (RPMI) media supplemented with 10% foetal bovine serum and 1% Pen/Strep in a humidified incubator at 37°C and 5% CO_2. The MeT-5A cell line was cultured in Dulbecco's modified Eagle's medium/F12 (1: 1) supplemented with 10% foetal bovine serum and 1% Pen/Strep in a humidified incubator at 37°C and 5% CO_2.

Proliferation assay after povidone-iodine treatment

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (CellTiter 96® Non-Radioactive Cell Proliferation Assay, Promega) was utilized to investigate the effects of PVP-I on proliferation and cell death in each cell line. Cells were seeded in media (3×10^4 cells/well) in triplicate in a 96-well

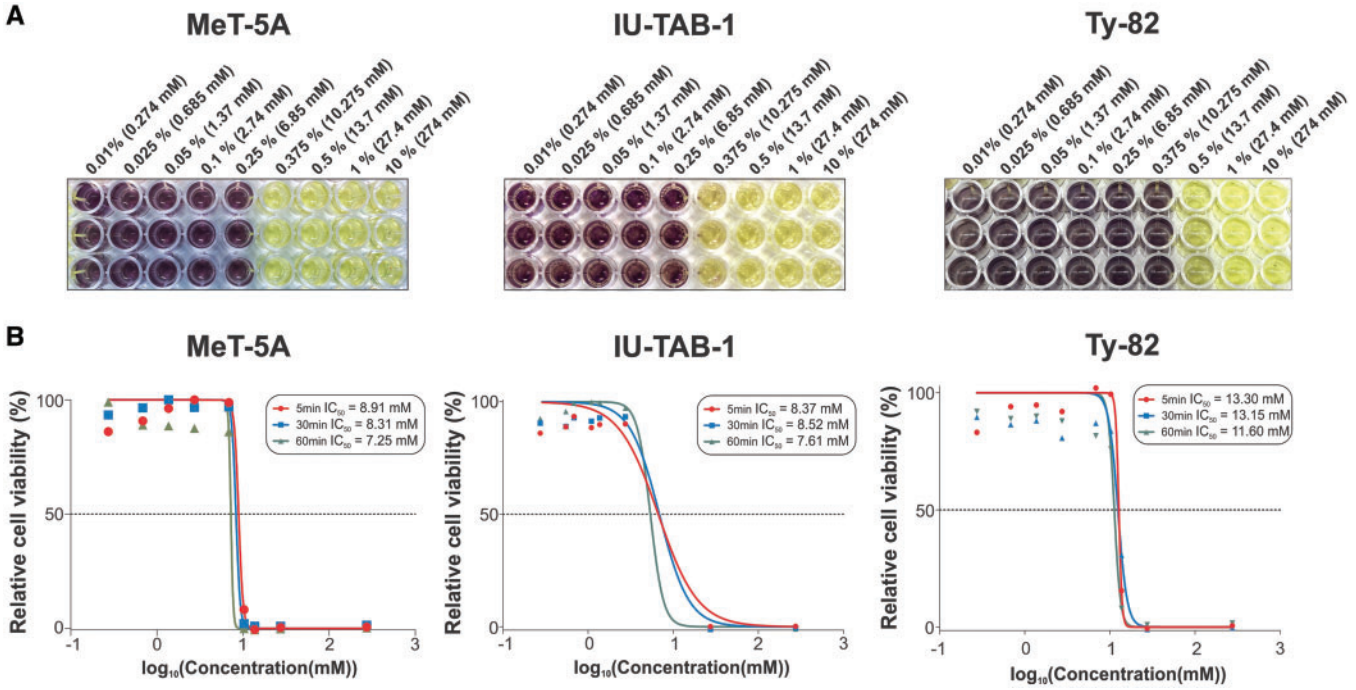

Figure 1: PVP-I kills TETs cells. (**A**) MTT assay was performed 24 h following a 5-min exposure to variable concentrations of PVP-I at 37°C. At PVP-I concentrations of 0.375%, 0.5%, 1% and 10%, cell death of MeT-5A and IU-TAB-1 was demonstrated by the lack of the purple formazan, and at PVP-I concentrations above 0.5%, Ty-82 cells induced cell death, demonstrated by the lack of purple formazan at these concentrations. (**B**) The IC_{50} value was determined for MeT-5A, IU-TAB-1 cells and Ty-82 cells. IC_{50}: half maximal inhibitory concentration; PVP-I: povidone-iodine; TET: thymic epithelial tumour.

plate and incubated overnight. PVP-I was added at 10 different concentrations (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.375, 0.5, 1 and 10%) at variable temperatures (37°C and 42°C). After 5, 30 or 60 min of PVP-I exposure, the cells were washed, and fresh media were added. MTT solution was added either 24 or 48 h later. Cells cultured in media alone were used as a positive control, and cell-free medium was used as a negative control. In the MTT assay, the yellow MTT tetrazole is reduced to purple formazan crystals by mitochondrial succinate dehydrogenase in live cells. Purple formazan crystals do not occur in dead cells. Following the addition of detergents such as dimethylsulphoxide (DMSO), these purple formazan crystals are solubilized. Cell viability was evaluated by spectrophotometric readings at 2 different wavelengths (570 and 650 nm), as specified by the manufacturer's instructions. All experiments were repeated at least 3 times.

Flow cytometry

Flow cytometry was performed to detect apoptotic activity and cell death. MeT-5A, IU-TAB-1 and Ty-82 cell lines (1×10^5 cells/ml) were seeded in a 6-well plate and treated with 0.1% and 1% concentrations of PVP-I for 24 h. Cells were resuspended in Maxpar Cell Staining Buffer (Cat#. 201068, Fluidigm) in individual 5-ml tubes for each sample. Apoptosis was evaluated by flow cytometry using FITC Annexin V, incorporating 7-AAD (Cat#. 640922, BioLegend, CA, USA) to evaluate cell death. Intracellular cleaved poly-ADP-ribose polymerase (cPARP) staining was evaluated using anti-human cPARP (Cat#. 558710, BD biosciences). After washing twice with 1 ml of Maxpar Cell Staining Buffer, samples were fixed with fixatives and permeabilized with permeabilization buffer (Cat#. 554723, BD biosciences) including 0.1% saponin. The samples are then stained with intracellular cPARP antibody for 30 min at room temperature and washed. Flow cytometry was performed with an LSR Fortessa cytometer (BD Biosciences) and 30 000–50 000 cells were counted for each measurement. Analysis was performed using the Flow Jo software (FlowJo, LLC.). Annexin V-positive cells were considered to represent the early stage of apoptosis (lower right quadrant of Annexin V and 7-AAD dot plots); Annexin V- and 7-AAD-positive cells were considered to indicate cell death (upper right quadrant). Annexin V- and 7-AAD-negative cells corresponded to the viable cell fraction (lower left quadrant).

Statistical analysis

All *in vitro* experiments were performed using at least triplicate wells. Differences in cell death rates among treatment groups were analysed by one-way analysis of variance with Dunnett's multiple comparisons using SPSS 24.0 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.), which was visualized with Prism® 5.0 (GraphPad Software, Inc., CA, USA). In all cases, $P < 0.05$ was considered statistically significant. IC_{50}, the half maximal inhibitory concentration, was calculated using Prism 5.0 (GraphPad Software, Inc., CA, USA).

RESULTS

Povidone-iodine kills thymic epithelial tumour cells

Treatment of IU-TAB-1 cells for 5 min with PVP-I concentrations of 0%, 0.01%, 0.025%, 0.05%, 0.1% and 0.25% demonstrated no

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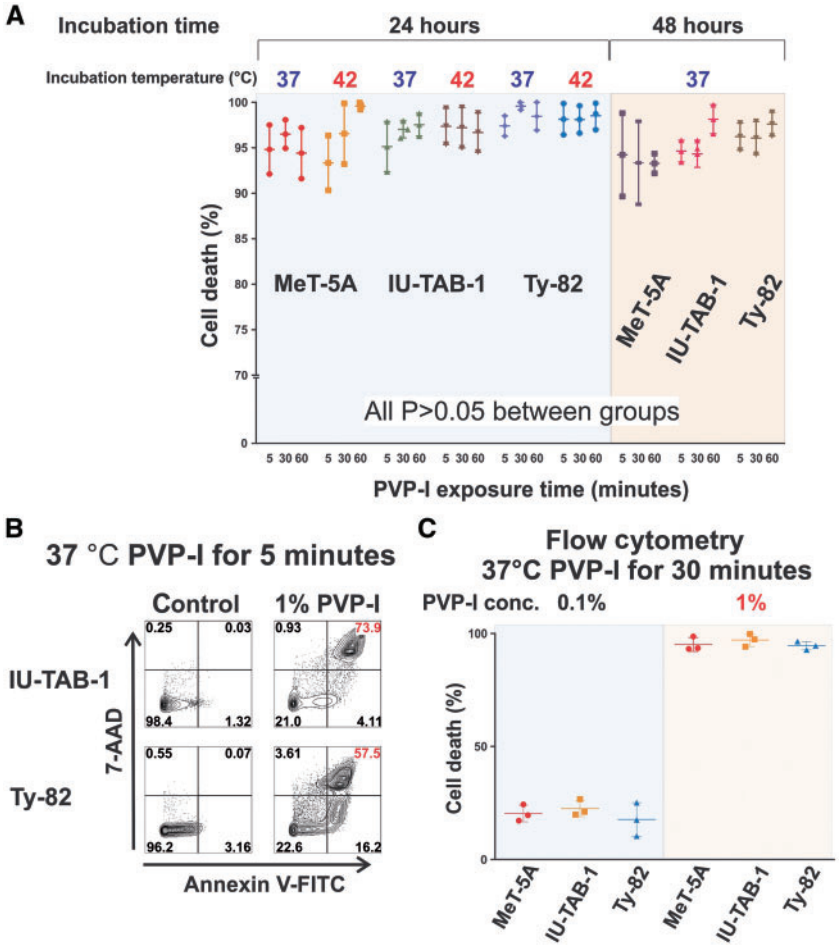

Figure 2: The cytotoxic effect of PVP-I is maximal at 30 min of exposure to 1% PVP-I and is independent of temperature. (**A**) MTT assays with 1% PVP-I in MeT-5A, IU-TAB-1 and Ty-82 cell lines according to temperature (37°C or 42°C), PVP-I exposure time (5, 30 or 60 min) and incubation times (24 or 48 h). (**B**) Apoptosis assay using flow cytometry immediately after exposure of 1% PVP-I for 5 min at 37°C in IU-TAB-1 and Ty-82 cell lines. (**C**) Cell death (Annexin-V+ 7-AAD+ cells) measured by flow cytometry following treatment of MeT-5A, IU-TAB-1 and Ty-82 cell lines for 30 min with 1% PVP-I at 37°C. PVP-I: povidone-iodine.

apparent cytotoxic effect at 24 h at 37°C. In these wells, the yellow tetrazole of the MTT assay was reduced to a purple formazan, which is driven by mitochondrial succinate dehydrogenase and indicates living cells (Fig. 1A). At PVP-I concentrations of 0.375%, 0.5%, 1% and 10%, cell death of MeT-5A and IU-TAB-1 was demonstrated by the lack of purple formazan. Similarly, the Ty-82 cells exposed to PVP-I in lower dilutions (0%, 0.01%, 0.025%, 0.05%, 0.1%, 0.25% and 0.375%) remained viable, whereas PVP-I concentrations above 0.5% induced cell death, demonstrated by the lack of purple formazan at these concentrations (Fig. 1A).

For all 3 cell lines, the extent of cell death was similar among PVP-I exposure times of 5, 30 and 60 min at 37°C. Concentration of PVP-I above 0.5% for as little as 5 min resulted in profound cell death, although Ty-82 (TC) was more resistant to killing by PVP-I than IU-TAB-1 (thymoma) and MeT-5A. The IC_{50} values for MeT-5A cells were 8.91 mM (0.32%) for 5 min, 8.31 mM (0.30%) for 30 min and 7.25 mM (0.26%) for 60 min. The IC_{50} values for IU-TAB-1 cells were 8.37 mM (0.30%) for 5 min, 8.52 mM (0.31%) for 30 min and 7.62 mM (0.28%) for 60 min. IC_{50} values for Ty-82 cells were 13.30 mM (0.49%) for 5 min, 13.15 mM (0.48%) for 30 min and 11.60 mM (0.42%) for 60 min (Fig. 1B).

The cytotoxic effect of povidone-iodine is maximal at 30 min of exposure to 1% povidone-iodine and is independent of temperature

PVP-I concentrations above 1% resulted in greater than 90% cell death in MeT-5A, IU-TAB-1 and Ty-82 cell lines regardless of temperature (37°C or 42°C), PVP-I exposure time (5, 30 or 60 min) and incubation times following PVP-I exposure (24 or 48 h) (Fig. 2A). The substantial cytotoxic effect of PVP-I on cell lines was consistently produced without any statistically significant differences between groups. Single-cell cytometric assays were performed to validate our MTT findings and to investigate the mechanism of cell death. Flow-cytometry-based apoptosis assays performed immediately after exposure of 1% PVP-I for 5 min at 37°C demonstrated 74% and 58% cell death (positive staining of both 7-AAD and Annexin V) in IU-TAB-1 and Ty-82 cell lines, respectively (Fig. 2B). These experiments further demonstrated that the majority of IU-TAB-1 and Ty-82 cells immediately experienced cell death (Annexin V^+/7-$AAD^{+)}$ after PVP-I treatment, bypassing necrotic death (Annexin V^-/7-AAD^+) and early apoptotic death (Annexin V^+/7-AAD^-). Increasing exposure time to 30 min of 1% PVP-I at 37°C resulted in increased cytotoxicity reaching 95.2%, 97.1% and 94.6% cell death in MeT-5A, IU-TAB-1 and Ty-82 cell lines, respectively (Fig. 2C).

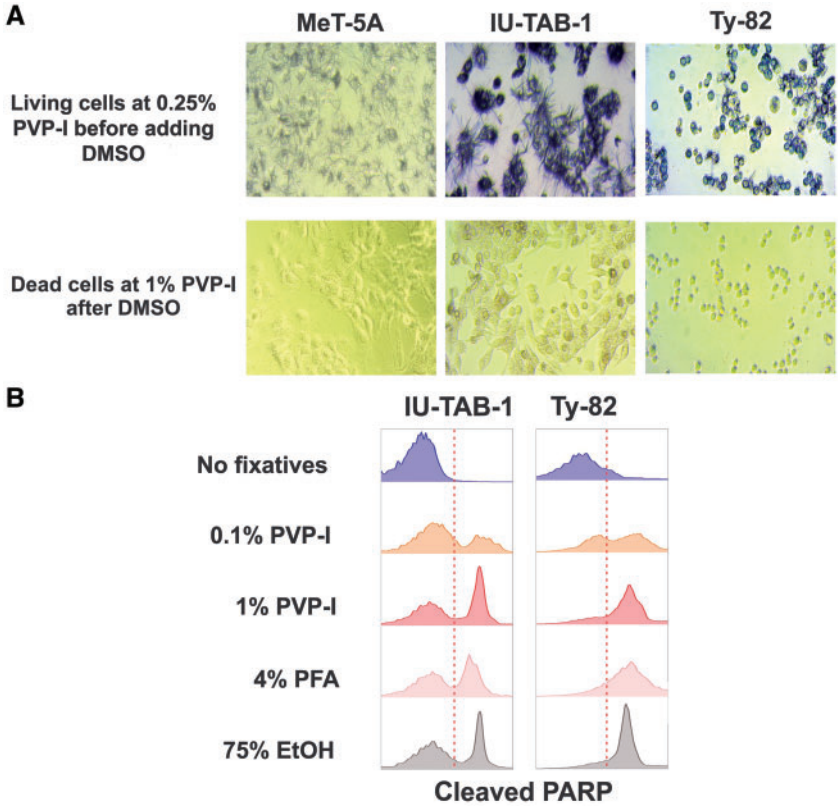

Figure 3: Povidone-iodine results in rapid killing of thymic epithelial tumour cells through cellular fixation. (**A**) Phase-contrast microscopic findings. Under low concentration of PVP-I, formazan crystals (purple) were visible in MeT-5A, IU-TAB-1 and Ty-82 cells evaluated in an MTT assay. Following the addition of DMSO to the MTT assay for purposeful destruction of the lipid bilayer structure of the cell membrane 4 h after incubation of cells in MTT solution, microscopy demonstrated that the cell structure was intact 5 min following 1% PVP-I treatment of thymic epithelial tumour cells. (**B**) Intracellular staining of cPARP for both IU-TAB-1 and Ty-82 after 1% PVP-I treatment showed to resemble cPARP staining following treatment with known fixatives, 4% paraformaldehyde (PFA) and 75% ethyl alcohol (EtOH). cPARP: cleaved poly-ADP-ribose polymerase; DMSO: dimethylsulphoxide; EtOH: ethyl alcohol; PFA: paraformaldehyde; PVP-I: povidone-iodine.

The mechanism of povidone-iodine-induced cytotoxicity on thymic epithelial tumour cells is cellular fixation

Because our flow cytometry assays suggested that TET cell death by PVP-I was not predominantly a result of apoptosis or necrosis, we evaluated cell morphology of PVP-I-treated TET cells with phase-contrast microscopy to investigate alternate modes of cell death. After exposure of MeT-5A, IU-TAB-1 and Ty-82 cells to low concentrations of PVP-I (<1%), microscopy demonstrated marked formation of intracellular formazan crystals following the addition of the MTT reagent (Fig. 3A). As standard in the MTT protocol, DMSO is added to destroy the lipid bilayer structure of the cell membrane 4 h after incubation of cells in MTT solution and thereby dissolve formazan crystals. Following the addition of DMSO to 1% PVP-I-treated TET cells during the MTT assay, contrast microscopy revealed that the cell structure of TET cells was intact, suggesting that cellular fixation was the predominant mechanism of cell death (Fig. 3A). While TET cells treated with low concentration of PVP-I were disrupted by DMSO, cells treated with 1% PVP-I were resistant to DMSO lysis at 1, 2 and 3 days. Resistance to cell lysis following exposure to 1% PVP-I was similar when HCl and isopropyl alcohol were substituted for DMSO as alternative solvents in MTT assays (data not shown). We next investigated the effects of 1% PVP-I on intracellular expression of cPARP, a cleaved form of PARP-1 (a nuclear chromatin-associated enzyme) that directs DNA-damaged cells to undergo a cell death and for which positive intracellular staining requires a fixation and permeabilization process. Exposure of IU-TAB-1 and Ty-82 cells to PVP-I resulted in a dose-dependent increase in intracellular cPARP expression, and exposure to 1% PVP-I resulted in an increase in cPARP expression resembling the expression following treatment with known fixatives, 4% paraformaldehyde and 75% ethyl alcohol (Fig. 3B).

DISCUSSION

We have demonstrated that PVP-I, at concentrations of 0.5% or greater, induce near-immediate cell death in human TET cells and normal mesothelial MeT-5A cells. In this *in vitro* system, an exposure time of 30 min resulted in 95% cell death of human thymoma and human TC cells and was independent of temperature. Further, our data indicate that the mechanism of TET cell death is cellular fixation.

It is well known that the microbicidal activity of PVP-I is due to its strong oxidizing effects of free iodine on amino (NH-), thiol (SH-) and phenolic hydroxyl (OH-) groups of amino acids and nucleotides. Additionally, iodine interacts strongly with the double bonds of unsaturated fatty acids in cell walls and cell organelle membranes [17, 18], and iodine atoms react with starch or glycogen by fitting into the helical coils of amylose to form the iodine–starch or glycogen complex, which is responsible for its sharp blue–black or brown–black color [19]. Previous studies in

human MPM, colorectal cancer, breast carcinoma, lung carcinoma and melanoma cell lines have suggested that tumour cell death by PVP-I occurs through apoptotic pathways [12–15, 20].

In contrast, our data in human TET cell lines support that cellular fixation is the primary mechanism of cell death from PVP-I, rather than apoptosis or necrosis. In support of our conclusion, we noted (i) the resistance of cell lysis against dissolving agents (indicating the maintenance of cell morphology), (ii) the similar intracellular staining of cPARP after PVP-I exposure to known intracellular fixatives and (iii) immediate cell death after PVP-I exposure (in contrast to the expected delayed death with necrosis or apoptosis). The discrepancy between our findings and previous reports may, in part, be explained by the timing of measurement of apoptosis markers. In apoptotic cell death, the time required between depolarization of the mitochondria and activation of the caspase cascade is approximately 30 min [21]. Rapid apoptosis can occur between 6 and 24 h after irradiation without cell cycle progression [22]. The late phase of apoptosis occurs after caspase activation and is represented by nuclear condensation and formation of the apoptotic bodies and occurs within as little as 3–4 h to 24–48 h [23]. Our data demonstrate substantial cell death of PVP-I-treated TET cells immediately after 5-min treatment with 1% PVP-I and provides compelling evidence that TET cell death from PVP-I does not occur through an apoptotic pathway. Further, our microscopy and intracellular cPARP staining data are in line with the report of Chou *et al.* [24] that demonstrated that treatment of human corneal fibroblast and human corneal epithelial cells with PVP-I at 0.1% or higher completely inhibited mitochondrial dehydrogenase and intracellular esterase activities through fixation, rather than apoptosis or necrosis, and that PVP-I-induced cytotoxicity is immediate, permanent and irreversible.

Although the use of PVP-I in multimodal treatment for Stage IVA TETs seems promising, there are important limitations to consider. Our data demonstrate that PVP-I is cytotoxic against human thymoma and human TC cells and, therefore, provides rationale for the use and study of intraoperative pleural PVP-I lavage following resection of TETs with pleural dissemination. Our data also demonstrate, however, that PVP-I has no target specificity, resulting in cell death of both human TET cells and a normal human mesothelial cell line. PVP-I when delivered intrapleurally, therefore, may have toxicity against normal host cells, and prolonged contact between PVP-I and viable tissue should probably be avoided. For example, the use of topical PVP-I has been reported to cause thyroid dysfunction in rare patients: an increase in serum levels of exogenous iodine has been shown both to inhibit thyroid hormone synthesis and to cause thyorotoxicosis [25]. The systemic absorption of iodine after pleurodesis with PVP-I appears safe and devoid of thyroid toxicity [26]; however, postoperative bilateral vision loss has been reported after thoracoscopic instillation of 10% PVP-I solution into the pleural space for prophylaxis against pneumothorax [27].

Limitations

Prior clinical studies have utilized hyperthermic (40–41°C) PVP-I lavage immediately following pleurectomy/decortication for Stage IVA TETs [11], although our data would suggest that a normothermic (37°C) PVP-I lavage, for 30 min, is sufficient to induce 95% TET cell death and can be considered clinically. Although hyperthermia did not improve the direct killing of TET cells in our *in vitro* study, the application of heated PVP-I in the operating room may still have beneficial effects. Previous *in vivo* studies have suggested that hyperthermia induces inhibition of intracellular repair mechanisms and results in increased tumour blood flow and consequent increased drug accumulation [28, 29]. Additionally, irrigation times less than 30 min can still lead to tremendous cell death and exposure times longer than that are likely unnecessary. One limitation of this study is that *in vivo* experiments were not performed to support *in vivo* efficacy and investigation of mechanism.

Based on our experimental findings, it seems reasonable to consider a 30-min normothermic 1% PVP-I lavage following cytoreductive surgery for Stage IVA TETs, which certainly would be best studied prospectively. Investigators of the International Thymic Malignancy Interest Group are currently in the process of designing a multi-institutional trial examining surgical resection of Stage IVA TETs combined with intraoperative instillation of heated PVP-I solution in the pleural space [30]. Our findings showcase PVP-I as a reasonable adjunct to control micrometastatic pleural disease in surgically resected Stage IVA TETs.

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Conflict of interest: none declared.

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