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Microsomal activation, and SH-SY5Y cell toxicity studies of tremetone and 6-hydroxytremetone isolated from rayless goldenrod (*Isocoma pluriflora)* and white snakeroot (*Agertina altissima)*, respectively

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ABSTRACT

This research compared the cytotoxic actions of the benzofuran ketone, tremetone in B16 murine melanoma cells to SH-SY5Y human neuroblastoma cells with an MTT assay. Tremetone was not cytotoxic in B16 cells. In SH-SY5Y cells, concentration-dependent tremetone cytotoxicity occurred without microsomal activation. No cytotoxicity was observed with 6-hydroxytremetone. This suggests that SH-SY5Y cells are a better model for the cytotoxic actions of tremetone and that tremetone is toxic without microsomal activation.

It has been reported that tremetone (Fig. 1A) a benzofuran ketone compound found in white snakeroot and rayless goldenrod requires activation by rat liver microsomes (RLM) to be cytotoxic (Beier et al., 1987, 1993; Beier and Norman, 1990). It has also been reported that tremetone is unstable and spontaneously converts to dehydrotremetone (Beier et al., 1987, 1993) Research conducted at this laboratory has shown that tremetone is stable for over 38 months when measured by repeated NMR analysis (Lee et al., 2012). The purpose uof our work was to; (1) compare the cytotoxic actions of tremetone in B16 murine melanoma cells and in SH-SY5Y human neuroblastoma cells with an MTT assay, (2) to determine if the actions of tremetone are concentration-dependent, and (3) if metabolism by RLM is needed to activate tremetone in order to be cytotoxic.

Tremetone is of interest because it is a putative toxin in white snakeroot and rayless goldenrod (Couch, 1927, 1930; Panter and James, 1990; Stegelmeier et al., 2012; Davis et al., 2013; Lee et al., 2015). Identifying the toxic principal(s) of white snakeroot is important because in addition to poisoning livestock by causing “trembles”, it also causes “milk sickness” in humans that drink the milk from dairy animals that have eaten the plants (Panter and James 1990). These diseases are associated with muscle weakness, muscle damage (as measured by elevated serum creatinine kinase activities), coma, and death (Kingsbury, 1964).

Research suggests that tremetone concentrations in plant material are not always associated with the toxic potential of white snakeroot (Davis et al., 2016, 2018), nor have goats been poisoned when dosed with tremetone containing extracts of white snakeroot adsorbed onto alfalfa (Davis et al., 2015). Davis et al. (2016) dosed a collection of white snakeroot containing 1.3 mg tremetone per g plant material to goats which resulted in creatinine kinase activities of 551 ± 508, versus, a second collection containing 0.8 mg tremetone per g plant material with increased creatinine kinase activities of 19,606 ± 27,558. The results of Davis et al. (2015, 2016) and other studies suggest that tremetone may not be the singular toxic principal(s) in white snakeroot.

For this work, cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured as described (Beier et al., 1987) using Flurobrite™ DMEM (Thermo Fisher Scientific, Waltham, MA). Cell culture reagents, RLM, and the Vybrant® MTT Cell Proliferation Assay Kit were obtained from Thermo Fisher. NADPH tetrasodium salt was obtained from Roche Diagnostics (Mannheim, Germany). For metabolic activation of the compounds, tremetone (isolated from rayless goldenrod, Lee et al., 2009), 6-hydroxytremetone (isolated from white snakeroot, Lee et al., 2010), and cyclophosphamide (Millipore Sigma, Burlington, MA) were incubated for 1 h with RLM as described by Beier et al. (1987). The microsomes were then removed with a 3000 nominal molecular weight limit Amicon Pro centrifugal filter unit (MilliporeSigma). Next, the cells were treated with the liver microsome exposed compounds or unexposed compounds as described in Beier et al. (1987), except for increasing the incubation time to overnight. We also included 4-hydroxycyclophosphamide (MilliporeSigma) which is the main active metabolite of cyclophosphamide (cyclophosphamide must be metabolized to be cytotoxic) as a reference compound (100%

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cytotoxicity). Cytotoxicity was measured with an MTT assay performed according to the kit instructions on a Molecular Devices Flexstation (San Jose, CA). Cell cytotoxicity was calculated as % cytotoxicity = (100 x (control – sample))/control, the control were cells allowed to grow in the absence of test compounds. The data were analyzed by two-way ANOVA and nonlinear regression with GraphPad Prism version 8.1.2 (http://WWW.graphpad.com) and significance was set at $P < 0.05$. The actions of 1 mM (B16 cells) and 100 μM (SH-SY5Y cells) 4-hydroxycyclophosphamide were used as 100 percent compound-induced cytotoxicity and the concentration-effect curves were normalized to those values (22 and 96% of untreated control, B16 and SH-SY5Y cells respectively).

Prior to this report, there were two descriptions of cell-based assays for benzofuran ketone toxicity by Beier et al. (1987, 1993). Beier et al. (1987) described a bioassay with murine melanoma B16 cells that pre-incubated benzofuran ketones with RLM and then exposed cells to RLM-incubated compounds that were thought to have undergone metabolism. In our research we first used B16 cells (Fig. 1B) in order to compare the tremetone isolated at this laboratory by Lee et al. (2009) to past reports by Beier et al. (1987, 1993) and an MTT assay to measure cytotoxicity.

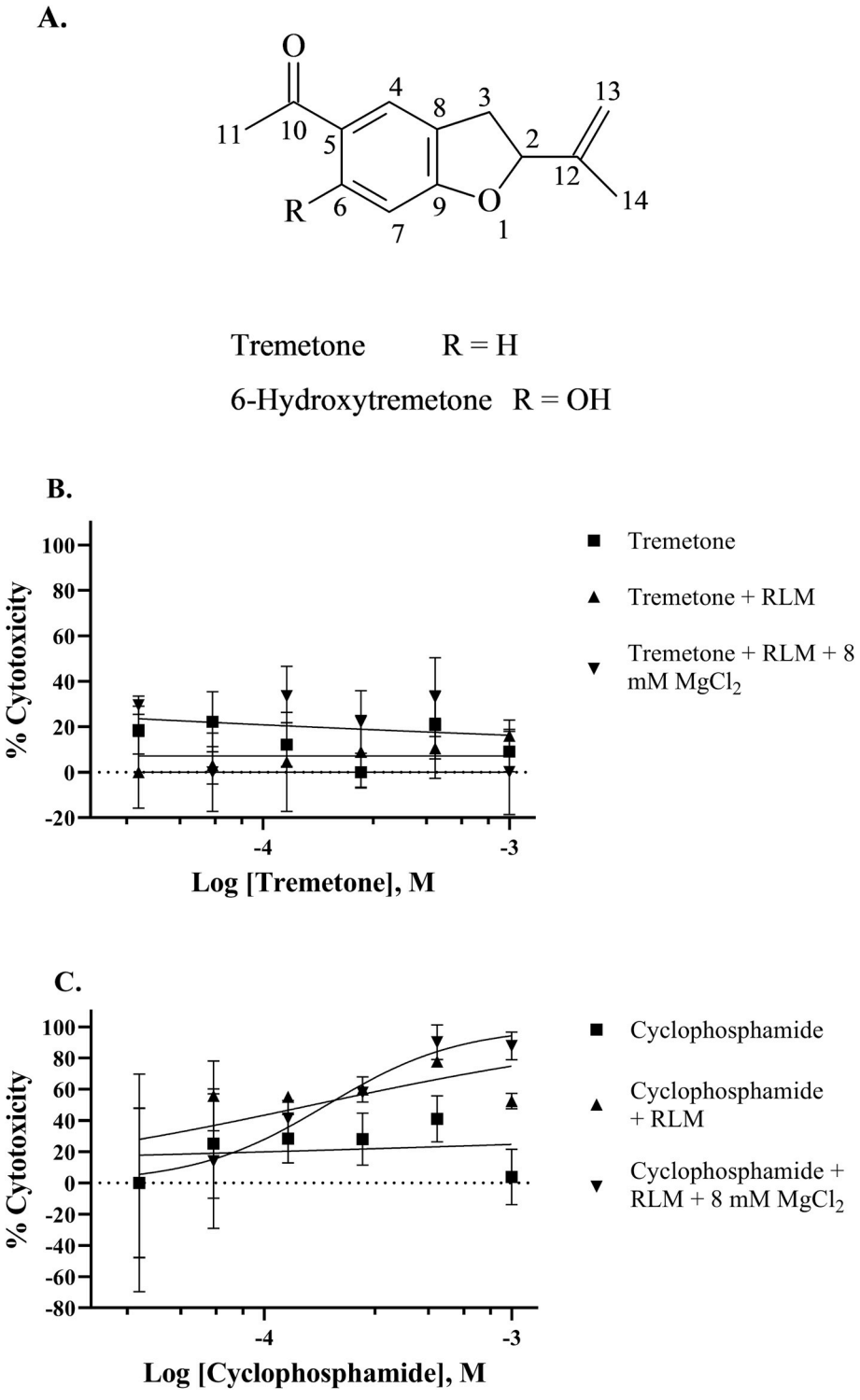

Fig. 1. Structures, and concentration-effect relationships of tremetone and cyclophosphamide in B16 cells. The chemical structures of tremetone and 6-hydroxytremetone (1A). Concentration-effect relationships with best-fit lines for the cytotoxic actions of tremetone (1B) and cyclophosphamide (1C) in B16 cells. In each experiment, the percent cytotoxicity of the B16 cells treated with the compound (tremetone or cyclophosphamide), the compound incubated with rat liver microsomes (RLM), or the compound incubated with RLM and 8 mM MgCl2 for 1 h, as described by Beier et al. (1987) is displayed in log10 M concentrations. Cytotoxicity was calculated as described in the text and displayed as a percentage of the maximal 4-hydroxycyclophosphamide cytotoxicity. Each datapoint for the tremetone experiments represents the mean ± SEM of six experiments of duplicate wells and each datapoint for the cyclophosphamide experiments represents the mean ± SEM of three experiments of duplicate wells.

Tremetone in B16 cells lacked concentration-dependent cytotoxicity (Fig. 1B) (P = 0.4057, concentration x treatment, two-way ANOVA). The RLM activated cyclophosphamide (Fig. 1C) in the presence of 8 mM $MgCl_2$ was cytotoxic in a concentration-dependent manner (P = 0.0066, concentration x treatment, two-way ANOVA; 50% inhibitory concentration (IC_{50}) = 179 μM, 95% confidence interval = 75–462 μM). $MgCl_2$ was included because divalent cations can act as cofactors that increase the catalytic activity of cytochrome P450 enzymes (Schrag and Wienkers, 2000). There was also some effect of the RLM on cyclophosphamide in the absence of 8 mM $MgCl_2$ (IC_{50} = 157 μM, 95% confidence interval not calculated). These results suggest that in B16 cells, tremetone is not cytotoxic while metabolized cyclophosphamide is cytotoxic. These results differ from those reported by Beier et al. (1987, 1993) which described acute toxicity in B16 cells after incubation with RLM. Beier et al. (1987, 1993).

In this study, SH-SY5Y cells were used to examine cytotoxicity of tremetone and 6-hydroxytremetone. This cell line has been used previously to investigate cytotoxicity of plant toxins (Green et al., 2010). In

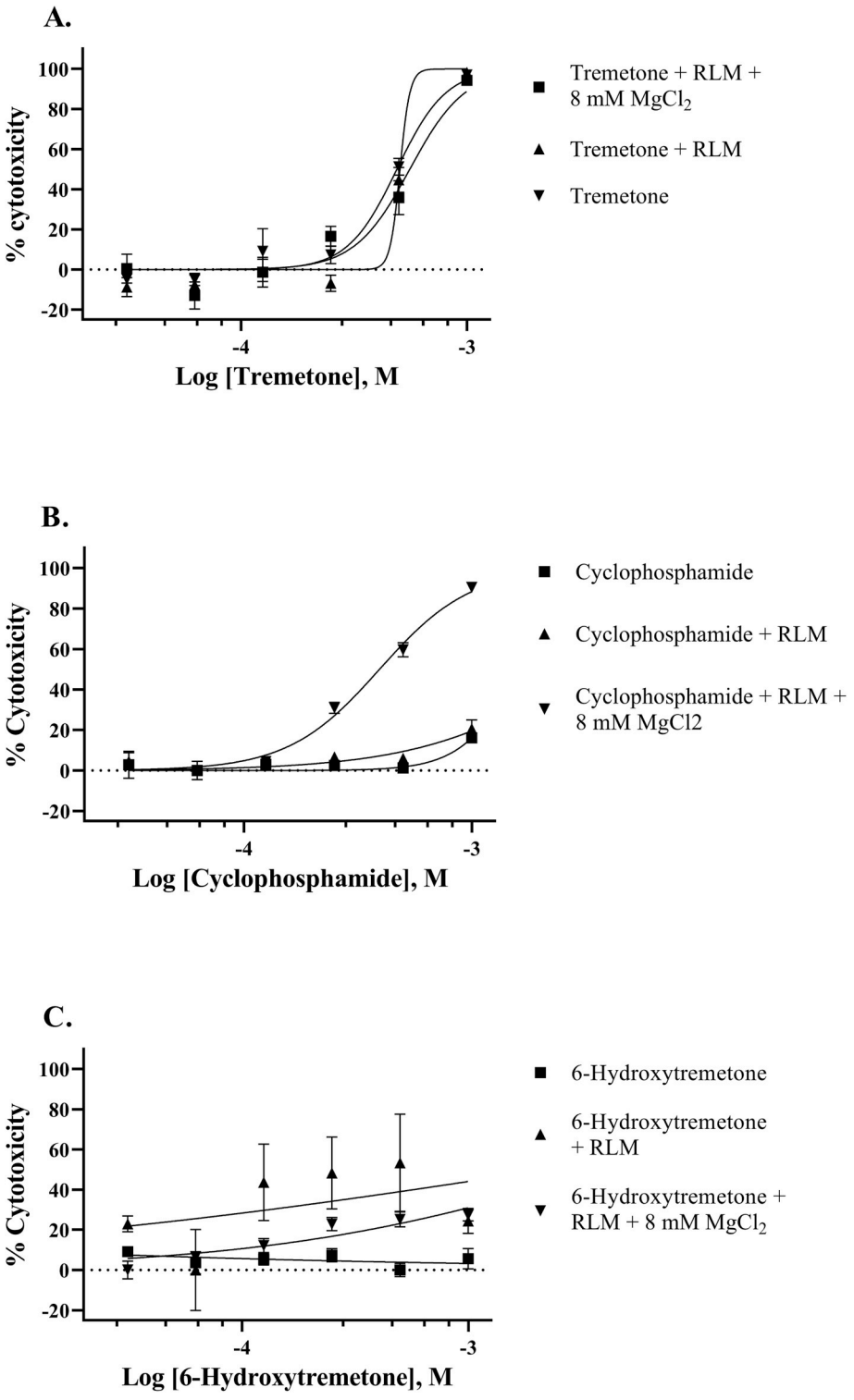

Fig. 2. Concentration-effect relationships with best-fit lines for the cytotoxic actions of tremetone (2A), cyclophosphamide (2B) and 6-hydroxytremetone (2C) as measured by an MTT assay in SH-SY5Y cells. In each experiment, the percent cytotoxicity of the SH-SY5Y cells treated with the compound (tremetone, cyclophosphamide, or 6-hydroxytremetone), the compound incubated with rat liver microsomes (RLM), or the compound incubated with RLM and 8 mM MgCl2 for 1 h, as described by Beier et al. (1987) is displayed in log10 M concentrations. Cytotoxicity was calculated as described in the text and displayed as a percentage of the maximal 4-hydroxycyclophosphamide cytotoxicity. Each datapoint for the tremetone and 6-hydroxytreme tone experiments represents the mean ± SEM of six experiments of duplicate wells and each datapoint for the cyclophosphamide experiments represents the mean ± SEM of three experiments of duplicate wells.

these cells (Fig. 2A–C), the actions of tremetone were concentration-dependent (P = 0.0011, concentration x treatment, two-way ANOVA) with or without RLM exposure (tremetone IC_{50} = 490 μM, 95% confidence interval 435–546 μM; tremetone and RLM IC_{50} = 505 μM, 95% confidence interval not calculated; tremetone, RLM and 8 mM $MgCl_2$ IC_{50} = 558 μM, 95% confidence interval 474–649 μM). Metabolized cyclophosphamide (Fig. 2b) was cytotoxic in a concentration-dependent manner that required $MgCl_2$ (P < 0.0001, concentration x treatment, two-way ANOVA; IC_{50} = 394 μM, 95% confidence interval = 356–435 μM). We also tested 6-hydroxytremetone in SH-SY5Y cells (Fig. 2c), it was much less effective as a cytotoxic agent and lacked concentration-dependency (P = 0.1402, concentration x treatment, two-way ANOVA). These results from experiments with SH-SY5Y cells suggest that tremetone does not require microsomal activation to be cytotoxic in SH-SY5Y cells in a concentration-dependent manner, and that tremetone was much more cytotoxic in SH-SY5Y cells than in B16 cells.

In this research, we compared the actions of tremetone in B16 murine melanoma cells to SH-SY5Y human neuroblastoma cells using an MTT assay to measure cytotoxicity. SH-SY5Y cells were a better model for measuring the concentration-dependent cytotoxic actions of tremetone. We also tested 6-hydroxytremetone in SH-SY5Y cells and its effects were not concentration-dependent. These results suggest that the presence of a hydroxyl group at the C-6 carbon (Fig. 1A) affects toxicity in SH-SY5Y cells. Finally, metabolism by RLM was not needed to activate the concentration-dependent actions of tremetone in SH-SY5Y cells demonstrating cytotoxicity in these cells without metabolism. This research with purified tremetone and 6-hydroxytremetone does support the hypothesis that tremetone is a toxin in white snakeroot and rayless goldenrod (Panter and James, 1990; Lee et al., 2015), but does not answer questions about tremetone concentrations in plant material and variable toxicity in goats (Davis et al., 2015, 2016). Further research is needed to identify the toxic principle(s) of white snakeroot and rayless goldenrod. We speculate that tremetone in combination with other benzofuran ketones found in white snakeroot and rayless goldenrod are required to cause trembles in goats and milk sickness in humans.

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References

Beier, R.C., Norman, J.O., 1990. The toxic factor in white snakeroot: identity, analysis and prevention. Vet. Hum. Toxicol. 32, 81–88.
Beier, R.C., Norman, J.O., Irvin, T.R., Witzel, D.A., 1987. Microsomal activation of constituents of white snakeroot (*Eupatorium rugosum* Houtt) to form toxic products. Am. J. Vet. Res. 48, 583–585.
Beier, R.C., Norman, J.O., Reagor, J.C., Rees, M.S., Mundy, B.P., 1993. Isolation of the major component in white snakeroot that is toxic after microsomal activation: possible explanation of sporadic toxicity of white snakeroot plants and extracts. J. Nat. Toxins 1, 286–293.
Couch, J.F., 1927. The toxic constituent of richweed or white snakeroot (*Eupatorium urticaefolium*). J. Agric. Res. 35, 547–576.
Couch, J.F., 1930. The toxic constituent of rayless goldenrod. J. Agric. Res. 40, 649–658.
Davis, T.Z., Green, B.T., Stegelmeier, B.L., Lee, S.T., Welch, K.D., Pfister, J.A., 2013. Physiological and serum biochemical changes associated with rayless goldenrod (*Isocoma pluriflora*) poisoning in goats. Toxicon 76, 247–254.
Davis, T.Z., Lee, S.T., Collett, M.G., Stegelmeier, B.L., Green, B.T., Buck, S.R., Pfister, J. A., 2015. Toxicity of white snakeroot (*Ageratina altissima*) and chemical extracts of white snakeroot in goats. J. Agric. Food Chem. 63, 2092–2097.
Davis, T.Z., Stegelmeier, B.L., Lee, S.T., Collett, M.G., Green, B.T., Pfister, J.A., Evans, T. J., Grum, D.S., Buck, S., 2016. White snakeroot poisoning in goats: variations in toxicity with different plant chemotypes. Res. Vet. Sci. 106, 29–36.
Davis, T.Z., Stegelmeier, B.L., Lee, S.T., Green, B.T., Chitko-McKown, C.G., 2018. Effect of grinding and long-term storage on the toxicity of white snakeroot (*Ageratina altissima*) in goats. Res. Vet. Sci. 118, 419–422.
Green, B.T., Lee, S.T., Panter, K.E., Welch, K.D., Cook, D., Pfister, J.A., Kem, W.R., 2010. Actions of piperidine alkaloid teratogens at fetal nicotinic acetylcholine receptors. Neurotoxicol. Teratol. 32, 383–390.
Kingsbury, J.M., 1964. Poisonous Plant of the United States and Canada. Prentice Hall, New Jersey.
Lee, S.T., Cook, D., Davis, T.Z., Gardner, D.R., Johnson, R.L., Stonecipher, C.A., 2015. A survey of tremetone, dehydrotremetone, and structurally related compounds in *Isocoma* spp. (goldenbush) in the southwestern United States. J. Agric. Food Chem. 63, 872–879.
Lee, S.T., Davis, T.Z., Gardner, D.R., Colegate, S.M., Cook, D., Green, B.T., Meyerholtz, K. A., Wilson, C.R., Stegelmeier, B.L., Evans, T.J., 2010. Tremetone and structurally related compounds in white snakeroot (*Ageratina altissima*): a plant associated with trembles and milk sickness. J. Agric. Food Chem. 58, 8560–8565.
Lee, S.T., Davis, T.Z., Gardner, D.R., Stegelmeier, B.L., Evans, T.J., 2009. Quantitative method for the measurement of three benzofuran ketones in rayless goldenrod (*Isocoma pluriflora*) and white snakeroot (*Ageratina altissima*) by high-performance liquid chromatography (HPLC). J. Agric. Food Chem. 57, 5639–5643.
Lee, S.T., Davis, T.Z., Cook, D., Stegelmeier, B.L., 2012. Evaluation of drying methods and toxicity of rayless goldenrod (*Isocoma pluriflora*) and white snakeroot (*Ageratina altissima*) in goats. J. Agric. Food Chem. 60, 4849–4853.
Panter, K.E., James, L.F., 1990. Natural plant toxicants in milk: a review. J. Anim. Sci. 68, 892–904.
Schrag, M.L., Wienkers, L.C., 2000. Topological alteration of the CYP3A4 active site by the divalent cation Mg(2+). Drug Metab. Dispos. 28, 1198–1201.
Stegelmeier, B.L., Davis, T.Z., Green, B.T., Lee, S.T., Hall, J.O., 2012. The resolution of rayless goldenrod (*Isocoma pluriflora*) poisoning in goats. Intl J oisonous Plant Res 2, 27–33.