

Interactions Between Nutraceutical Supplements and Standard Acute Myeloid Leukemia Chemotherapeutics

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Abstract - PURPOSE: Concomitant use of nutraceuticals with chemotherapy is very common. Cancer patients self-medicate to relieve the side effects associated with chemotherapy, improve disease outcome and to regain control of their medical care. However, there is limited empirical evidence on potential drug-nutraceutical interactions and their resulting effect on chemotherapy efficacy. **METHOD:** To investigate drug-nutraceutical interactions we created and screened a library of commonly used nutraceuticals for their modulatory effects on the activity of cytarabine and daunorubicin, two primary chemotherapeutics used to treat acute myeloid leukemia (AML). Combination screening was performed in 3 AML cell lines (OCI-AML2, KG1a and U937) using the MTS viability assay. Lead compounds were validated using with the Annexin V/ Propidium iodide assay and CalcuSyn drug combination software. **RESULTS:** We identified zinc as a nutraceutical that enhanced AML chemotherapy efficacy with combination index (CI) values of 0.649, 0.632 and 0.615 at EC 25, 50 and 75, respectively; CI values <0.9, >1.1 or between 0.9-1.1 denote statistical synergy, antagonism or additivity, respectively. In contrast, we show that echinacea hindered AML chemotherapy efficacy by significantly reducing the ability of cytarabine to induce cell death. **CONCLUSION:** Given the positive and negative effects of nutraceuticals, patients undergoing chemotherapy must consult with their oncologist before consuming over-the-counter supplements.

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INTRODUCTION

At some point after diagnosis, 60-80% of patients suffering from a hematological malignancy consumed an over-the-counter dietary supplement (i.e., nutraceutical, or food-derived bioactive compound) (1). Although the development of methotrexate involved understanding the negative impact of folic acid on acute lymphoblastic leukemia (ALL) cell growth and ALL patient survival, there remains limited clinical evidence supporting or refuting nutraceutical use by cancer patients. Thus, given the importance of understanding the role of nutraceuticals in cancer chemotherapy, we performed a drug combination screen to identify nutraceuticals that enhanced and hindered the efficacy of cytarabine and daunorubicin, two primary therapeutics used in the treatment of acute myeloid leukemia (AML).

MATERIALS AND METHODS

Reagents

Nutraceuticals were generously provided by Jamieson Laboratories Inc. (Windsor, ON, Canada) and cytarabine (AraC; Tocris Bioscience; Bristol, UK) and daunorubicin (Da; Tocris Bioscience; Bristol, UK) were purchased and reconstituted according to the manufacturer's protocol. Stock solutions were diluted in phosphate buffered saline (PBS), aliquoted and stored at -20°C.

Cell Culture

AML (OCI-AML2, KG1a, U937) cell lines were cultured (5% CO₂ at 37°C) in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies; Grand Island, NY) supplemented with 10% fetal calf serum (Seradigm) and antibiotics (100 units/mL of streptomycin and 100 µg/mL of penicillin; Sigma Chemical).

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Cell growth and viability

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) reduction assay (Promega; Wisconsin) and Annexin V and propidium iodide (ANN/PI) staining (Biovision; Mountainview, CA) were used to measure cell growth and viability according to the manufacturer's protocols and as previously described (2).

Nutraceutical screen

The unique nutraceutical library was created and screened similar to previously described methods (2). Briefly, AML cells (1.5×10^4 /well) were seeded in 96-well plates. After seeding, cells were treated with aliquots (1 mg/ml final concentration) of library compound alone or in combination with $3 \mu\text{M}$ cytarabine and $0.05 \mu\text{M}$ daunorubicin, which correspond to their EC₅₀ values (data not shown). After 72 hours, cell growth and viability were measured by the MTS assay.

Statistical and combination index analysis

Unless otherwise stated, the results are presented as mean \pm SD. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, USA). $p < 0.05$ was accepted as being statistically significant. CalcuSyn software was used to analyze and generate combination index (CI) values, as previously described (3). The software assesses the combined effect of two drugs under fixed molar ratios and generates CI values as a function of cell death. These graphical representations display the potential synergy, antagonism, or additivity of two drugs over a wide range of fixed molar ratio doses.

RESULTS**Nutraceutical screen identifies potential interaction between zinc and cytarabine**

The nutraceutical library was screened against 3 AML cell lines following a 72 hour incubation period in the presence or absence of an AML chemotherapeutic. EC₅₀ values for cytarabine and daunorubicin were incubated with 1 mg/ml of an individual nutraceutical and viability was assessed by the MTS assay. Heat map analysis showed that zinc (compound 20) had the greatest effect on enhancing cytarabine and daunorubicin's efficacy (Figure 1A). Zinc alone had an EC₅₀ of ~ 0.012 mg/ml when validated with the ANN/PI assay (Figure 1B, left panel). The ability of zinc to

synergistically interact with the clinical therapeutics was next tested using CalcuSyn, where CI values < 0.9 , > 1.1 or between 0.9-1.1 denote statistical synergy, antagonism or additivity, respectively (3). The combination of cytarabine and zinc was synergistic (CI values of 0.649, 0.632 and 0.615 at EC 25, 50 and 75) (Figure 1C, left panel) while concomitant treatment with daunorubicin displayed an additive relationship (Figure 1C, right panel).

Nutraceutical screen identifies Echinacea as a compound that hinders cytarabine and daunorubicin activity.

Heat map analysis of our drug combination screen showed that compound 23 and echinacea (compound 21), had the greatest effect on hindering cytarabine and daunorubicin's cytotoxicity (Figure 1A). However, compound 23 was a false positive and was not assessed further (validation results not shown). Echinacea's inhibitory activity was confirmed in secondary assays and since it was not cytotoxic to AML cells at the concentrations used (Figure 1D), we were unable to use CalcuSyn software to determine CI values (i.e., it requires EC₅₀ values). Thus, we incubated echinacea with cytarabine or daunorubicin in equal molar ratio concentrations, as described in the methods, and used the ANN/PI assay to measure cell viability. Echinacea significantly inhibited cytarabine and daunorubicin's cytotoxicity in AML cells (Figure 1D).

DISCUSSION

A high throughput screen of a nutraceutical library has shown that zinc is able to enhance and echinacea is able to hinder the efficacy of AML chemotherapy. Given the high rate of nutraceutical consumption, this information is critical for accurate patient recommendations.

Zinc is an essential trace element in humans and supplementation (2mg/kg/day for 60 days) in children and adolescents with acute leukemia was effective in relieving chemotherapy-associated side effects (weight loss, malnutrition, nausea, and vomiting) (4). This study demonstrated the safety and physiological benefit of zinc supplementation but did not evaluate supplementation effects on chemotherapy efficacy. Marginal zinc deficiencies can have deleterious effects on DNA integrity and repair (5) and zinc supplementation in zinc deficient patients has been shown to increase DNA repair to normal levels (5).

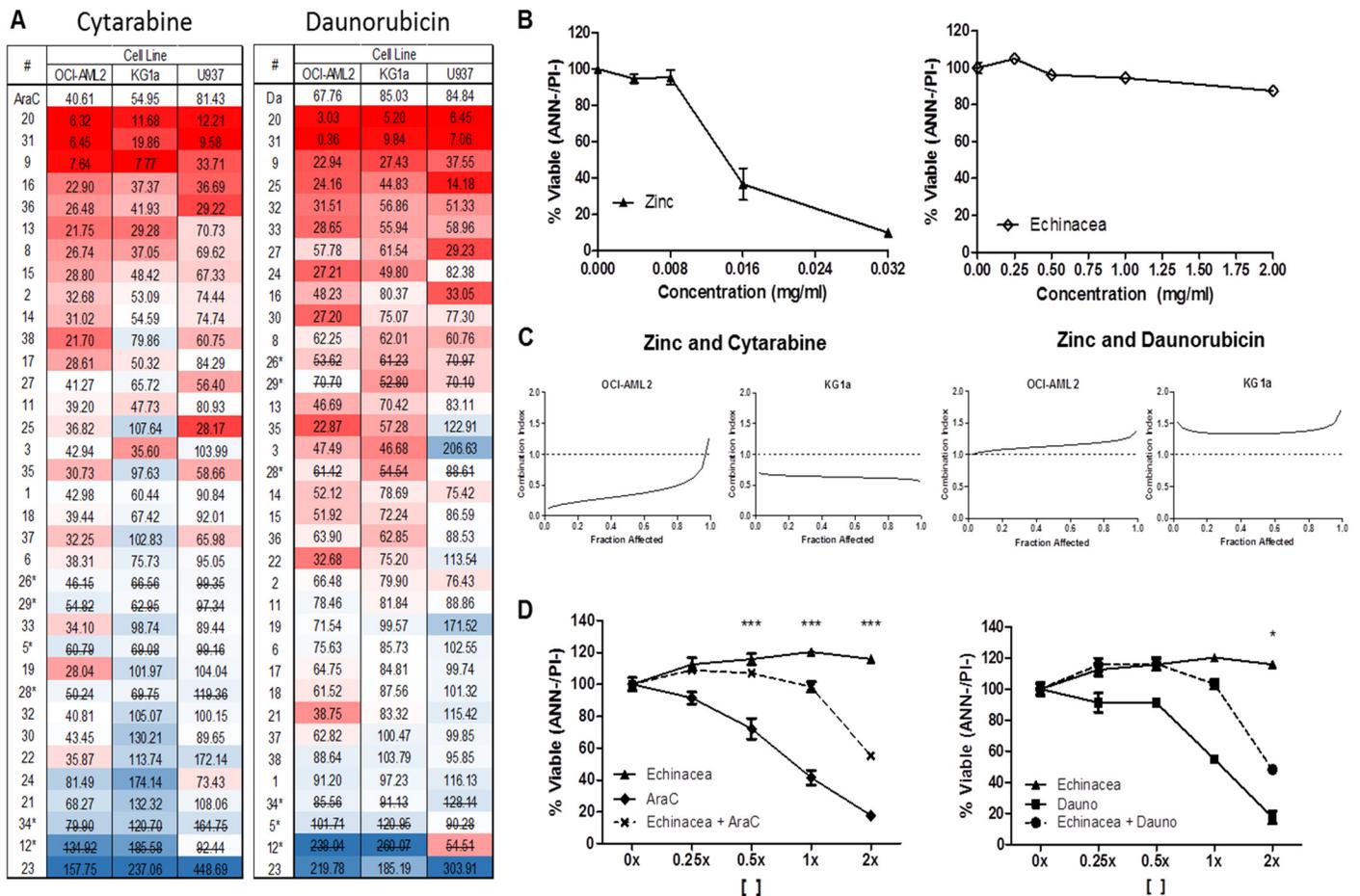


Figure 1. Combination screening identifies nutraceuticals that interact with common AML therapeutics. (A) Heat map showing the effect on viability when combining compounds from our nutraceutical library with either Cytarabine (AraC) or Daunorubicin (Da). Values are the average ($n=3$) of a 72 hour MTS assay measuring cell viability in 3 different AML cell lines (OCI-AML2, KG1a, and U937). Red colour indicates decreased cell growth and viability and blue colour indicates increased cell growth. All values are relative to the viability of cells treated with AraC or Da alone. Note: compounds that changed the colour of the media thereby affecting our assay are indicated by a strikethrough in the heatmap and were not further assessed. (B) Annexin V/PI cell viability assay assessing dose-response of zinc and echinacea in AML cells. (C) Combination index (CI) versus cell death (i.e., fraction affected) graphs demonstrating the effect of zinc in combination with cytarabine or daunorubicin. CI values were determined using the CalcuSyn software as outlined in the methods. (D) Combination graphs demonstrating negative interactions between echinacea with cytarabine or daunorubicin after 72 hr using the ANN/PI cell viability assay in AML cells. Data are the mean percentage of viability cells \pm SD from three experiments. * $p<0.05$, *** $p<0.0005$.

Thus, when in combination with cytarabine, which can impart its anti-cancer activity by incorporating metabolites into DNA during DNA synthesis, zinc may be potentiating cytarabine's activity by increasing the rate of DNA repair thereby incorporating more arabinofuranosylcytosine triphosphate (i.e., araCTP, the active form of AraC) into DNA. None-the-less, future detailed mechanistic studies are needed to confirm the exact mechanism of this synergistic interaction.

Zinc is tightly regulated and supplementation will only marginally increase total zinc plasma levels. Supplementation to alleviate chemotherapy side effects, were demonstrated at plasma concentrations of 1.0 ± 0.07 mg/L zinc after 60 days (4). In our study, zinc was used in the form of zinc gluconate (MW 455.685 g/mol) in which zinc is found at 12.9% (as formulated by Jamieson). In our study, *in vitro* synergistic interactions were seen at zinc concentrations of 4.0mg/L (Figure 1C).

Therefore, in order to achieve relevant physiological levels, zinc would need to be administered systemically with cytarabine, as oral supplementation may not achieve the plasma concentrations required to impart synergy. Recently it was shown that nanoparticle zinc (ZnO-NPs) administered intravenously in rats resulted in zinc plasma levels of 1000 mg/L with no associated toxicity (6). None-the-less, future pharmacokinetic studies with oral zinc supplementation or intravenous zinc are needed to support clinical efficacy.

Through this screen we have also demonstrated that Echinacea, among the most purchased nutraceuticals, hindered the efficacy of AML chemotherapy. Echinacea has been shown to increase the cellular concentration of CYP3A, a potent drug metabolizer, which could decrease plasma concentrations of anticancer drugs that are CYP3A substrates such as cytarabine (7, 8). Therefore, echinacea could be hindering cytarabine by inducing CYP3A enzymes. In addition, lymphocytes harvested from echinacea supplemented mice showed up-regulation of the anti-apoptotic Bcl-2 protein (9). This provides evidence that echinacea can have anti-apoptotic *in vivo* modulatory effects, which could be of great importance to patient outcome as Bcl-2 is commonly overexpressed in AML. Cytarabine and daunorubicin can cause AML cell apoptosis through this pathway and thus, echinacea may interfere with these chemotherapeutics through Bcl-2 modifications.

Due to the multiple formulations and sources of echinacea in supplements, it is difficult to define physiologically relevant doses and accurately assess pharmacokinetics. In this study, a maximum of 2 mg/ml final concentration of *Echinacea Purpurea* (Jamieson Inc., *Echinacea Purpurea* (4:1) extract 87.5 mg root, NPN# 80010071) was used, which is similar to previous reports (10). Multiple studies administered echinacea at high doses in humans (i.e., 1500 mg/day for 28 days) and mice (30 – 100 mg/kg for 14 days) with no signs of toxicity (7) (9). Therefore, given echinacea's ability to reduce chemotherapy efficacy, cancer patients undergoing chemotherapy should avoid echinacea until further studies can confirm its safety.

In summary, cytarabine and daunorubicin's activity were enhanced by the nutraceutical zinc and hindered by echinacea. These results provide seminal evidence that define the role of

nutraceuticals in AML chemotherapy and provide the basis for future studies that will lead to important clinical recommendations for AML patients.

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AUTHORSHIP CONTRIBUTIONS

LA and PAS performed experiments, analyzed data and wrote the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

Jamieson Laboratories Inc. provided the nutraceuticals free of charge, however, provided no other means of financial contribution. The authors declare no conflict of interest.

REFERENCES

1. Ben-Arye E, Attias S, Tadmor T, Schiff E. Herbs in hemato-oncological care: an evidence-based review of data on efficacy, safety, and drug interactions. *Leuk Lymphoma*. 2010;51(8):1414-23. Epub 2010/06/10.
2. Lee, E. A., Angka, L., Rota, S. G., Hanlon, T., Mitchell, A., Hurren, R., Wang, X. M., Gronda, M., Boyaci, E., Bojko, B., Minden, M., Sriskanthadevan, S., Datti, A., Wrana, J. L., Edginton, A., Pawliszyn, J., Joseph, J. W., Quadrilatero, J., Schimmer, A. D., and Spagnuolo, P. A. (2015) Targeting Mitochondria with Avocatin B Induces Selective Leukemia Cell Death, *Cancer research* 75, 2478-2488.
3. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Advances in enzyme regulation*. 1984;22:27-55. Epub 1984/01/01.
4. Consolo LZ, Melnikov P, Consolo FZ, Nascimento VA, Pontes JC. Zinc supplementation in children and adolescents with acute leukemia. *Eur J Clin Nutr*. 2013;67(10):1056-9. Epub 2013/08/22.
5. Song Y, Leonard SW, Traber MG, Ho E. Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses, and DNA repair in rats. *J Nutr*. 2009;139(9):1626-31. Epub 2009/07/25.
6. Amara S, Ben-Slama I, Mrad I, Rihane N, Jeljeli M, El-Mir L, et al. Acute exposure to zinc oxide

- nanoparticles does not affect the cognitive capacity and neurotransmitters levels in adult rats. *Nanotoxicology*. 2014;8 Suppl 1:208-15. Epub 2014/02/15.
7. Penzak SR, Robertson SM, Hunt JD, Chairez C, Malati CY, Alfaro RM, et al. Echinacea purpurea significantly induces cytochrome P450 3A activity but does not alter lopinavir-ritonavir exposure in healthy subjects. *Pharmacotherapy*. 2010;30(8):797-805. Epub 2010/07/27.
 8. Colburn DE, Giles FJ, Oladovich D, Smith JA. In vitro evaluation of cytochrome P450-mediated drug interactions between cytarabine, idarubicin, itraconazole and caspofungin. *Hematology*. 2004;9(3):217-21. Epub 2004/06/19.
 9. Di Carlo G, Nuzzo I, Capasso R, Sanges MR, Galdiero E, Capasso F, et al. Modulation of apoptosis in mice treated with Echinacea and St. John's wort. *Pharmacol Res*. 2003;48(3):273-7. Epub 2003/07/16.
 10. Tsai YL, Chiu CC, Yi-Fu Chen J, Chan KC, Lin SD. Cytotoxic effects of Echinacea purpurea flower extracts and cichoric acid on human colon cancer cells through induction of apoptosis. *J Ethnopharmacol*. 2012;143(3):914-9. Epub 2012/09/14.