Translational Studies of Protandim® as a Candidate Nutraceutical Approach to Treating Ovarian Cancer

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Dedication

I would like to dedicate my thesis to my beloved parents, and my aunt.

Abstract

Background and Rationale: Use of nutraceutical approaches are rapidly increasing in cancer patients. We encountered a recurrent ovarian cancer patient who incurred durable tumor regression and decreasing CA-125 coincident with initiation of the nutraceutical Protandim®, a combination of five phytochemical extracts (ashwagandha, bacopa, green tea, milk thistle, turmeric). Preclinical studies were undertaken to investigate Protandim® and Protandim® constituent anticancer effects and underlying mechanism(s).

Methods: *In vitro* and *in vivo* ovarian cancer cell line models were used to assess Protandim® and Protandim® constituent effects. Colony forming assays, Hoechst nuclear and Trypan exclusion staining, immunoblotting, and flow cytometric methods were used to assess growth inhibitory, cytotoxic, apoptotic/necrotic effects as well as to preliminarily assess involved mechanism(s) of action. Combined synergistic/additive/antagonistic effects of Protandim® components were assessed by the methods of Chou and Talalay; an *ex vivo* myeloma patient model system was used to assess cancer selectivity. *In vivo* studies assessed Protandim® tolerability and toxicity over a wide oral dosage range.

Results: Anticancer effects of Protandim® and its constituent extracts were demonstrated, with induction of necrotic – not apoptotic - morphological cell death. *Ex vivo* assays demonstrated Protandim® to selectively kill freshly collected patient myeloma cells, relatively sparing paired patient normal bone marrow cells, indicating anticancer-selectivity. Immunoblotting and flow cytometric experiments indicated that Protandim® induced increased levels of cellular reactive oxygen species (ROS). Similar cytotoxic effects in wild-type- and Rho-MOLT4 cells (absence of mitochondria function) indicated non-mitochondrial mediated ROS induction by Protandim®. Assessment of the combined effects of Protandim® constituents primarily demonstrated antagonism or additivity. *In vivo* mouse studies demonstrated no Protandim® toxicities when compounded in diet during 43 days of feeding.

Conclusions: Protandim® and some of its ingredients (green tea and turmeric) have promising activity in ovarian cancer models, associated with induction of non-mitochondrial mediated-ROS and necrosis. Moreover, Protandim® is well-tolerated in mice, and has anti-cancer selectivity when assess in a myeloma/normal cell *ex vivo* model. Further investigations to more specifically assess molecular mechanism and *in vivo* efficacy are presently underway, in anticipation of eventual translation to therapeutic human clinical trials in ovarian cancer.

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List of abbreviations

A2780 cell line, ovarian cancer cell line

Akt, protein kinase B (serine/threonine-specific protein kinase)

ALT, alanine- amino-transferase

ANOVA, analysis of variance

AST, aspartate-amino-transferase

BCA, bicinchoninic acid assay

BCS, body condition scoring

BUN, blood urea nitrogen

C13, ovarian cancer cell line with platin-resistant

C200, ovarian cancer cell line

CA-125, cancer antigen 125, or carcinoma antigen 125, or carbohydrate antigen 125

CD138, Syndecan 1; a protein which in human is encoded by the syndecan (SDC) 1 gene

CI, Combination Index

CMH2DCFDA, 5,6-chloromethyl-20, 70-dichlorodihydrofluorescein diacetate

Cr, creatinine

CT, computed tomography

D, dose

DHE, dihydroethidium

Dm, median dose effect

DMEM, Dulbecco's Modified Eagle's Medium

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DRI, dose reduction index

Duox, dual oxidases

ECs, endothelia cells

ED50, effective dose 50; minimum effective dose for 50% of the population

EGCG, (-)-epigallocatechin-3-gallate

ER, endoplasmic reticulum

fa, affected fraction

FACS, Fluorescence-Activated Cell Sorting analysis

FAD, Flavin Adenine Dinucleotide

FasR, Fas receptor

fu, unaffected fraction

FY, fiscal year

G1, gap 1

G2, gap 2

GCLC, glutamate-cysteine ligase catalytic

GCLM, glutamate-cysteine ligase modifier

GST, glutathione S-transferase

H_2O_2, hydrogen peroxide

HCAEC, human coronary artery endothelial cells

HO-1, heme oxidase 1

IACUC, Institutional Animal Care and Use Committee

IC_{50}, inhibit cellular proliferation by 50%

ip, intraperitoneal

IRB, international review board

m, the slope of the median-effect plot

M, mitosis

MAb, monoclonal antibody

MEM, Minimum Essential Medium

Mito-ETC, mitochondrial electron transport chain

NADPH oxidases (NOX), Nicotinamide Adenine Dinucleotide Phosphate Oxidase

NFE2L2 (Nrf2), Nuclear factor (erythroid-derived 2)-like 2

NOXO, NADPH oxidase organizer

Nqo1, nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1

$O2^{*-}$, superoxide anion

OV (202, 2008) cell line, ovarian cancer cell line

$p22^{phox}$, Neutrophil cytochrome b 22 kilo Dalton polypeptide, alpha polypeptide type of cytochrome b-245

p38MAPK, p38 mitogen-activated protein kinase

$p40^{phox}$, 40 kilo Dalton cytosolic subunit of neutrophil cytosolic factor 4

$p47^{phox}$, 47 kilo Dalton cytosolic subunit of neutrophil NADPH oxidase (Neutrophil cytosolic factor 1)

p53, phosphoprotein p53, or cellular tumor antigen p53, or tumor suppressor p53

$p67^{phox}$, 67 kilo Dalton cytosolic subunit of the multi-protein complex (Neutrophil cytosolic factor 2)

PARP1, poly (adenosine diphosphate ribose) polymerase-1

PBS, phosphate-buffered saline

PCD, program cell death

PEO cell line, estrogen receptor positive Human ovarian cancer cell line

PI3-kinase, phosphatidylinositide 3-kinase

PKCδ, protein kinase C delta type

platin, cisplatin

r, the correlation coefficient

Rac GTPases, Rho family of GTPases; a family of small (~21 kilo Dalton) signaling G protein and is a subfamily of the Ras superfamily

Rho-MOLT4 cell line, acute lymphoblastic leukemia cell line with mitochondrial respiratory chain nonfunctioning

RIP, receptor interacting protein

RMPI media, Roswell Park Memorial Institute media

ROS, reactive oxygen species

S, synthesis

SD, standard deviation

SDS, sodium dodecyl sulfate

SE, standard error

SEM, standard error of the mean

SKOV3 cell line, Sloan-Kettering HER2 3+ Ovarian Cancer cell line

SKOV3TR cell line, Sloan-Kettering HER2 3+ Ovarian Cancer cell line with Taxol resistant

SOD, superoxide dismutase

T1, the first stage of translational research

t-BHP, tert-butyl hydroperoxide

TNFR, tumor necrosis factor receptor

UGT, uridine diphosphate- glucuronosyltransferase

VEGF, vascular epithelial growth factor

WT-MOLT4 cell line, wild type of acute lymphoblastic leukemia cell line

Chapter 1:
Introduction

Introduction

Epithelial ovarian cancer is the deadliest of all cancers of the female reproductive system and the fifth most common cause of cancer mortality in women (1). Every 24 minutes marks another diagnosis of ovarian cancer in the United States; a woman's lifetime risk of developing invasive ovarian cancer is 1 in 72 (1-3). In 2013, the American Cancer Society estimates that 22,240 new cases will be diagnosed and an 14,030 women will die of ovarian cancer in the United States alone (1). In spite of the National Cancer Institute's (NCI) investment of $110.8 million in fiscal year (FY) 2011 for ovarian cancer research (4-5), treatment outcomes for ovarian cancer remain poor. In particular, overall 5-year survival is < 35% and "cure" rates have unfortunately not substantively improved in decades. Consequently, there is need for additional therapeutic innovation to further improve not only response durations and "cure" rates, but also to attain lessened adverse effects.

Patient-based Anecdotal Protandim® Experience

The genesis of this study stems from a 64 year-old women diagnosed with recurrent fallopian tube carcinoma with peritoneal metastasis and a rising of the serum tumor marker, CA-125. She declined standard salvage chemotherapy, and instead, on her own volition, initiated nutraceutical therapy with Protandim®. Interestingly, this patient incurred durable clinical improvement in terms reduction in the size of her peritoneal lesions, and declining CA-125 without apparent toxicity. This anecdotal experience triggered our interest in assessing the anticancer effects of Protandim® and raised the question of whether it might have clinical application beyond this anecdotal experience.

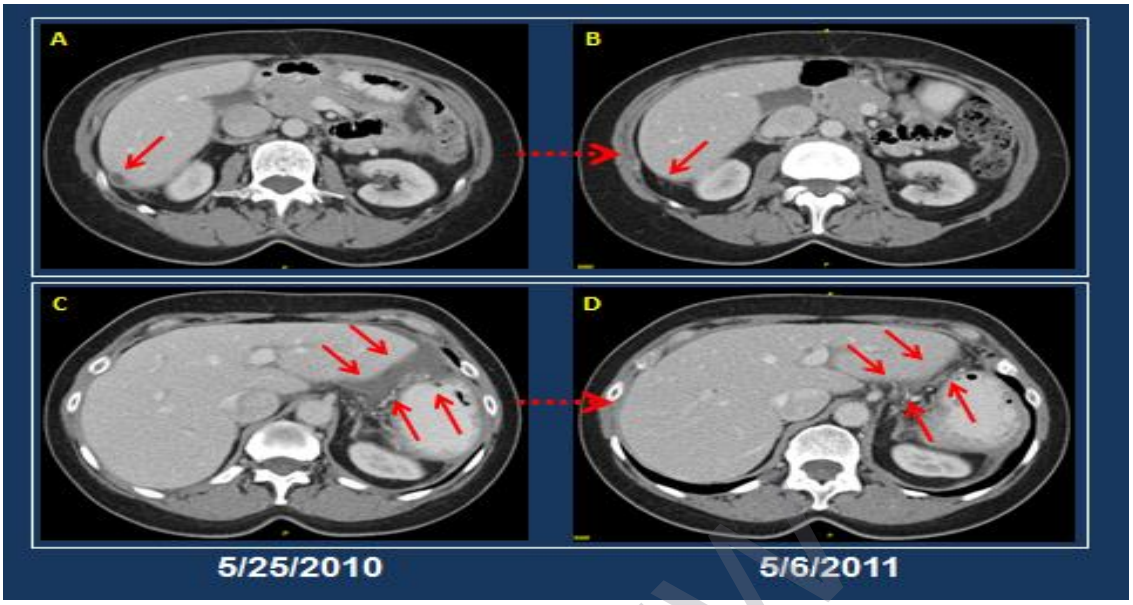

Figure 1: A 64 year-old female patient, treated for peritoneal metastasis: A and C: CT upper abdomen before taking Protandim® that showed 2 areas of peritoneal fluid/metastasis; B and D: CT upper abdomen 12 months after taking Protandim® that the two areas were significantly decreased in size (near complete response).

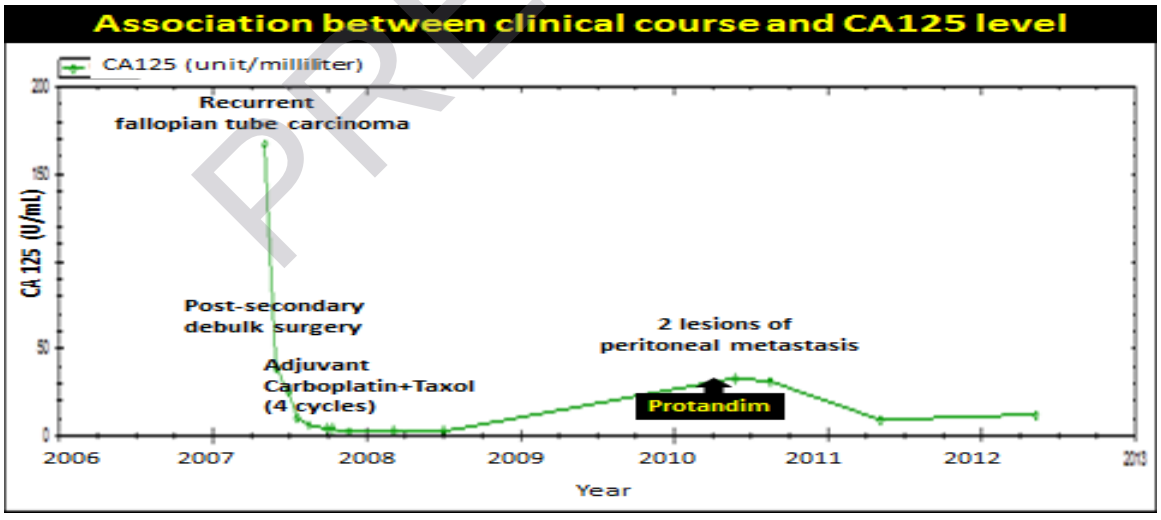

Figure 2. Changes of the serum CA-125 before and after taking Protandim®. CA-125 declined after Protandim® use for 3 months, attaining normal range (< 35 unit/milliliter) at 6 months of Protandim® use.