HORMONE EPIMERS REGULATE ER STRESS AND CORE REGULATORY GENES: NETWORK ANALYSIS WITH APPLICATIONS TO GLIOMA AND CHRONIC PRESSURE ULCERS

Thomas L. Shaak
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd
Part of the Life Sciences Commons

Downloaded from https://scholarscompass.vcu.edu/etd/531

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
HORMONE EPIMERS REGULATE ER STRESS AND CORE REGULATORY GENES: NETWORK ANALYSIS WITH APPLICATIONS TO GLIOMA AND CHRONIC PRESSURE ULCERS

A Dissertation submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy at Virginia Commonwealth University

by

THOMAS L. SHAAK, LtCol, USAF, BSC
B.S. Microbiology, Pennsylvania State University, 1990
M.S. Health Systems Administration, University of Medicine and Dentistry of New Jersey, 2003
M.S. Integrated Life Science, Medical University of South Carolina, 2005

Director: Dr. Robert F. Diegelmann, PhD., Professor of Biochemistry

Virginia Commonwealth University
Richmond, Virginia
August 2013
ACKNOWLEDGEMENT

The author would like to thank several people and without them I do not think I would be here now. First, Dr. Douglas Wear, Dr. Mina Izadjoo, PhD., Dr. Sue Cross, PhD, Dr. Mohammed Alavi, PhD, Dr. Hyung Kim, DVM, PhD. all of whom inspired me when I thought I was beyond inspiration. Along that same line I would like to thank my sister Caren Butera, who provided an amazing amount of inspiration and support in many ways. Second, I would like to thank Dr. Robert F. Diegelmann and Dr. Kevin R. Ward for introducing me to a very special group of researchers who are involved in the cutting edge of science and medicine. I would like to make a special acknowledgement for Dr. Robert F. Diegelmann whose depth and breadth in wound healing is endless. I would like to thank him for accepting me into his lab, introducing me to many professional colleagues and pointing me in the right direction when I need it. I would also like to make a special acknowledgement to Dr. Roger M. Loria, his generosity in allowing me to work with the androstene hormones, tireless guidance and mentorship on all aspects of science, the many hours he spent discussing complex ideas have resulted in the best educational experience that I have ever had. I would like to thank the other members of my committee Dr. Danail Bonchev and Dr. John Ryan for guiding my project and introducing me to concepts which have enhanced my skills. I would also like to thank my parents Dr. Thomas A. Shaak, DDS and Mary A. Shaak for their support and for my kids, Jonathon and Katelyn who, have worked very hard on their programs at Texas A&M and made being father very easy and this work possible.
TABLE OF CONTENTS

LIST OF TABLES............................................................................................................iv
LIST OF FIGURES............................................................................................................v
LIST OF ABBREVIATIONS..............................................................................................vi
ABSTRACT......................................................................................................................viii

CHAPTER 1: Introduction.................................................................................................1

   Historical Perspective: Discovery of the anti-inflammatory effects of corticosteroids............................................................................................................1

   Hypothalamus Pituitary Adrenal Axis........................................................................2

   Dehydroepiandrosterone (DHEA)...............................................................................3

   Anticancer effects of DHEA.......................................................................................4

   Immune effects and DHEA.......................................................................................5

   Anti-autoimmune effects and DHEA.........................................................................6

   Neural effects and DHEA..........................................................................................6

   DHEA, stress and the anti-glucocorticoid response..................................................7

   DHEA as a precursor to androstene hormones.........................................................8

   Δ5-androstene-3β, 17β-diol (17β-AED) and Δ5-androstene 3β, 17β-diol
   (17βAET) Metabolism...............................................................................................9
DHEA, Δ5-androstene-3β, 17β-diol (17β-AED), Δ5-androstene-3β, 7β, 17β-triol (17β-AET), immunity and antiglucocorticoid activity………………..10

Transcriptional Effects of 17β-AED and 17β-AET…………………………………..12

DHEA, 17β-AED, 17β-AET and the estrogen/androgen Receptors…………………14

Δ5-androstene-3β, 17α-diol (17α-AED)………………………………………………17

Overview…………………………………………………………………………………..22

CHAPTER 2: Structural Stereochemistry of Androstene Hormones Determines Interactions with Human Androgen, Estrogen, and Glucocorticoid Receptors…………………………………………………………………………….24

Introduction………………………………………………………………………………24

Methods………………………………………………………………………………….25

Nuclear Receptor Transactivation Assays……………………………………………25

Preparation of Stock Hormone Solutions……………………………………………26

Mass Spectrometry……………………………………………………………………27

Cellular Uptake of Androstene Hormones and Normalization of Transactivation Assay Results………………………………………………………….28

Metabolism of Androstene Hormones………………………………………………28

Statistical Analysis………………………………………………………………………29
Results and Discussion..........................................................................................29

Androstene hormone structures...........................................................................29

Androstene Hormone Activation of the Human AR receptor..............................31

Androstene Hormone Activation of the human ERβ and ERα Receptors..............33

Androstene Hormone Activation of the Human Glucocorticoid (GR) Receptor..........................................................36

Summary...............................................................................................................42

CHAPTER 3: Androstene Hormone Epimers Regulate ER Stress and Core Regulatory Genes in Human T98G Glioma Cells........44

Introduction.......................................................................................................44

Methodology....................................................................................................51

Cell culture.......................................................................................................51

Androstene Hormone Controls..........................................................................51

The Human Signal Transduction Pathway Finder Microarray............................51

IPA Network Generation and Analysis..............................................................54

Network Generation........................................................................................54

Network Score Statistics..................................................................................54

Results and Discussion.....................................................................................55

The Human Signal Transduction Pathway Finder Microarray............................55

Network Analysis............................................................................................57

Target Genes...................................................................................................57

General Discussion............................................................................................59
### Table of Contents

**CHAPTER 4: Pressure Ulcers in Patients with Spinal Cord Injury; Microarray and Network Analysis**

- Introduction .......................................................... 73
- Materials and Methods ............................................. 75
- Protection of Human Subjects ..................................... 75
- Recruitment and consent procedures .......................... 75
- Specimens ............................................................... 76
- Total RNA Extraction from Skin Tissues ....................... 76
- Microarray analysis using Illumina Human-6 BeadChip arrays ........................................ 77
- Microarray data analysis ............................................ 79
- Network Analysis ..................................................... 79
- Network Core Analysis ............................................. 79
- Network Statistics (Score) ........................................... 79
- Core regulator Analysis of Chronic Wounds ................. 80
- Results and Discussion ............................................ 81
- Summary .................................................................. 100

**CHAPTER 5: General Discussion and Overall Conclusion** ............................................. 104

**REFERENCES** .......................................................... 117

**APPENDIX 4A** .................................................................. 142

**APPENDIX 4B** .................................................................. 144

**APPENDIX 4C** .................................................................. 146
LIST OF TABLES

Table 1: Relative Androgenicity of Androstene Hormones.................................33
Table 2: Relative Estrogenicity of Androstene Hormones.................................36
Table 3: Biological Function of 17α-AED and 17β-AED....................................49
Table 4: 17α-AED/17β-AED Target Genes in T98G Glioma Cells.........................58
Table 5: Whole Genome Microarray Network Analysis:
  Ubiquitin C-centered Networks........................................................................78
Table 6: Functions of Genes Associated with UBC in Normal Skin Controls and
  Chronic Pressure Ulcers of Spinal Cord Injury Patients..............................102
LIST OF FIGURES

Figure 1. The structures of the Androstene Hormones.................................................................30

Figure 2. Androstene Hormone Activation of the Human Androgen Receptor..........................32

Figure 3. Androstene Hormone Activation of the Human Estrogen Receptor Beta..................34

Figure 4. $17\beta$-AED Activation of the Human Estrogen Receptor Alpha.................................35

Figure 5. Dexamethasone or Androstene Hormone Activation of the Human Glucocorticoid Receptor.................................................................................................................................37

Figure 6. Androstene Hormones and Dexamethasone Activation of the Human GR Receptor..................................................................................................................................................39

Figure 7. Androstene Hormone and Dexamethasone Activation of the Human GR Receptor in the presence of Cyproterone Acetate.........................................................41

Figure 8. Contrasting Effects on Cell Morphology of T98Glioma cells treated with $17\alpha$-AED or $17\beta$-AED..................................................................................................................................................46

Figure 9: Structure-function relationship of the $17$ hydroxyl position of the chemically identical androstenediol epimers, $17\alpha$-AED and $17\beta$-AED results in opposing biological functions...........................................47

Figure 10: Human signal Transduction Pathway Finder Microarray..........................................53

Figure 11: Contrasting Effects on Gene Expression in T98Glioma cells treated with $17\alpha$-AED or $17\beta$-AED...........................................................................................................................................56

Figure 12: General Mechanism of Autophagy Induction by $17\alpha$-AED........................................60
Figure 13: Stress Induced Responses Directed by 17α-AED and 17β-AED

Figure 14: BRCA-1 directed, p53 dependent transcriptional response induced by 17β-AED

Figure 15: Opposing Regulation of p53 Expression Induced by 17α-AED or 17β-AED through WNT1, AKT and GSK3β

Figure 16: Core Regulatory Unit Governing Cell Cycle Arrest, Apoptosis, Inhibition of Angiogenesis and Metastasis and DNA Repair

Figure 17: T98G Gene Mutations in 3 Critical Glioblastoma Signaling Pathways

Figure 18: Network schematic of genes that are associated with UBC in the A) the normal skin network #1 or B) the chronic pressure ulcer network #1

Figure 19: UBAP1 Related Core Master Transcriptional Regulator Network
LIST OF ABBREVIATIONS

($\alpha$) = alpha position

$17\beta$-AED = $\Delta^5$-androstene-3$\beta$, 17$\beta$-diol

$17\alpha$-AED = $\Delta^5$-androstene-3$\beta$, 17$\alpha$-diol

$17\beta$-AET = $\Delta^5$-androstene-3$\beta$, 7$\beta$, 17$\beta$-triol

Akt = v-akt murine thymoma viral oncogene homolog, protein kinase B

Androstene Hormones = $17\beta$-AED, $17\alpha$-AED, $17\beta$-AET, DHEA

Androstene Hormone Metabolites = $17\beta$-AED, $17\alpha$-AED, $17\beta$-AET

AP-1 = Activating Protein 1

AR = Androgen Receptor

ARD = Ankyrin Repeat Domain

ATF6 = Activating Transcription Factor 6

($\beta$) = beta position

BCL2 = B-cell CLL/lymphoma 2

BCR-ABL = Break Point Cluster Region Protein-Abelson

Beclin-1 = beclin-1, autophagy related
BEX = Brain Expressed X-linked
BIRC2 = baculoviral IAP repeat containing 2
BMP2 = bone morphogenic protein 2
BRCA1 = Breast Cancer 1 early onset
CTSD = Cathepsin D
C8orf76 = Chromosome 8 Open Reading Frame 76
c-MYC = V-myc myelocytomatosis viral oncogenes homologue(Avian)
CCL2 = Chemokine (C-C motif) ligand 2
CDK2 = Cyclin-dependent kinase 2
CDKN1A = Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN2D = Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)
CERCAM = Cerebral endothelial cell adhesion Molecule
CHOP = CCAAT/enhancer-binding protein homologous protein
CPU = Chronic Pressure Ulcer
CLIP3 = CAP-GLY domain containing linker protein 3
ColGALT1 = Collagen Galactose Transferase 1
ColGALT2 = Collagen Galactose
CSN2 = Casein beta
CYLD = Cylindromatosis tumor syndrome
CMV = Cytomegalovirus

CtBP = C-terminal binding protein 1

CtIP = Retinoblastoma binding protein 8

DEF8 = Differentially Expressed in FDCP homologue (mouse)

DHEA = Dehydroepiandrosterone

DNA = Deoxyribonucleic Acid

DUB = Deubiquinating enzyme

DUSP-1 = Dual Specificity Phosphatase 1

E1 = Ubiquitin Activating Enzyme

E2 = Ubiquitin Conjugating Enzyme

E3 = Ubiquitin E3 Ligase

ELAVL1 = ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1

E2F = E2F transcription factor group

EGFR = Epidermal Growth Factor Receptor

ER = Endoplasmic Reticulum
ERα = Estrogen receptor alpha

ERβ = Estrogen receptor beta

ERAD = Endoplasmic Reticulum Associated Degradation

ERN1 = Endoplasmic reticulum to nucleus signaling 1

ESCRT1 = Endosomal Sorting Complexes Required for Transport 1

ERK1/2 = Extracellular Signaling Related Kinase 1/2

ESR1 = Estrogen Receptor Alpha 1

FBA = Fbox associated protein

FBX = Fbox

FBXO = Fbox subfamily member

GADD45A = Growth Arrest and DNA Damage Inducible Alpha

GAPDH = Glyceraldehyde phosphate dehydrogenase

GCR = Glucocorticoid receptor

GPX = Glutathione peroxidase

GR = glucocorticoid receptor

GYS1 = Glycogen synthase 1 (muscle)

GCN2 = General control non-derepressable 2

GCR = Glucocorticoid receptor

GERAD = Glycoprotein endoplasmic reticulum associated degradation
LMF2 = Lipase maturation factor 2

MHC I = Major histocompatibility class 1

MMGT1 = Membrane magnesium transporter 1

NFkBIA = Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha

mEMC = Mammalian ER membrane complex

mRNP = Messenger ribonucleoprotein

NAB2 = NGFI-A binding protein 2 (EGR-1 binding protein 2)

NCCRP-1 = Nonspecific cytotoxic cell receptor protein-1

ODC1 = Ornithine decarboxylase 1

p300/CBP = E1A binding protein/p300/CREB Binding Protein

P53 = Tumor protein 53

PDGF = Platelet derived growth factor

PERK = PRKR-like endoplasmic reticulum kinase

PI3K = Phosphoinositide-3- kinase

PI3KC3 = Phosphoinositide-3-kinase catalytic subunit type III

PMEPAI = Prostate transmembrane protein-androgen induced

PKR = Protein kinase, interferon-inducible double stranded RNA dependent activator

Rb = Retinoblastoma protein

RILPL1 = Rab interacting lysosomal
RIP1 = Receptor (TNFRSF)-interacting serine-threonine kinase 1

RNA = Ribonucleic Acid

RNF = Ring finger

RNF24 = Ring finger protein 24

RNF145 = Ring finger protein 145

SECISBP2L = SECIS binding protein 2 homologue (S. cerevisiae)

SCI = Spinal cord injury homologue (S. cerevisiae)

SCF = Skp1, Cul1, Fbox and Rbx-1

SECISBP2L = SECIS binding protein 2-like

SIAH1 = Siah E3 ubiquitin ligase 1

SIRT1 = Sirtuin 1

SLC22A17 = Solute carrier family 22

SWI-SNF = Switch/sucrose non-fermentable

SQSTM1 = Sequestosome 1

STAT1 = Signal transducer and activator of transcription 1, 91kDa

Th1 = T helper Type 1

Th2 = T helper Type

THUMPD1 = THUMP domain containing 1

TNFα = Tumor necrosis factor alpha
TNFRSF1 = Tumor necrosis factor receptor 1

TP53I3 = tumor protein 53 inducible protein 3

TRC8 = Ring finger protein 139

TRPC = Transient receptor potential channel

TsiL = T-cell secretion of interleukin

TSG101 = Tumor susceptibility gene 101

UBAP1 = Ubiquitin associated protein 1

UBC = Ubiquitin C

Vps28 = Vacuolar protein sorting 28

Vps34 = Phosphatidylinositol 3-kinase, catalytic subunit type 3

Vps37A = Vacuolar protein sorting 37A
ABSTRACT

HORMONE EPIMERS REGULATE ER STRESS AND CORE REGULATORY GENES: NETWORK ANALYSIS WITH APPLICATIONS TO GLIOMA AND CHRONIC PRESSURE ULCERS

THOMAS LEE SHAAK, PhD

A Dissertation submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2013

Director: Dr. Robert F. Diegelmann, PhD., Professor of Biochemistry

DHEA has been determined to have medically significant activity and is the parent compound to the more active metabolites; 17α-AED, 17β-AED and 17β-AET, which exhibit strong biological activity that has been attributed to androgenic, estrogenic or anti-glucocorticoid activity in vivo and in vitro. This study compared DHEA, 17α-AED, 17β-AED and 17β-AET for their ability to activate the human AR, ER and GR receptors and determine the relative androgenicity, estrogenicity and glucocorticoid activity. The results show that, at the receptor level, these androstene hormones are weak AR and even weaker ER activators. Direct androstene hormone
activation of the human AR, ERα, and ERβ may not be essential for their biological function. Similarly, these hormones indirectly activated the human GR receptor; only in the presence of high dexamethasone concentrations. These results underscore the major difference between androstene hormone interactions with these nuclear receptors.

17β-AED and 17α-AED, androstene epimers that produce either survival or death, were utilized to treat T98G Glioblastoma cells. We identified 26 genes oppositely regulated by 17β-AED and 17α-AED to directly affect the cellular life or death decision. Network analysis demonstrated that these 26 genes are essential to regulating three critical Glioblastoma pathways. This report, for the first time, demonstrates that naturally occurring, chemically identical adrenal hormones (17β-AED or 17α-AED) direct a cellular life or death decision through contrasting modulation of identical signaling pathways and core regulators.

Chronic pressure ulcers represent a significant health problem and are characterized by hypoxia, bacterial infection, repetitive ischemia/reperfusion and altered cellular and systemic stress responses. Whole genome microarray analysis was utilized in conjunction with IPA® premiere networking software to analyze chronic wound edge tissue. IPA® network analysis identified Ubiquitin C (UBC) as the most significant network. Sixteen (16) ubiquitin C associated genes were identified to be different in the chronic pressure ulcer and normal skin control. Targeted network analysis associated core regulators to 8 UBC associated genes that are unique to chronic pressure ulcers. The identification of these genes will allow the establishment of more effective treatments for Spinal Cord Injury (SCI) patients with chronic pressure ulcers.
CHAPTER 1: Introduction

*Historical Perspective: Discovery of the anti-inflammatory effects of corticosteroids*

The effects of adrenal insufficiency was first discovered in 1855 by Thomas Addison [1]. In 1939, Butenandt received a Nobel Prize in chemistry for his work on steroid hormones and steroidogenesis [2]. This work and the work of others at this time period began the study of the steroid producing endocrine glands. Shortly thereafter, Kendall, Hench and Reichstein were awarded the Nobel Prize for the discovery of the anti-inflammatory effect of cortisol in rheumatoid arthritis patients marking the first time an endogenous hormone was discovered with significant anti-inflammatory effects [3]. This work sparked the clinical use of corticosteroids as long-term therapeutics for chronic inflammatory diseases. The beneficial effects of corticosteroids, however, was overshadowed by association with immune suppression and risk of infection, osteoporosis, thinning of the hair, stria formation, growth inhibition, hematopoietic abnormalities, psychological changes and delayed wound healing. Glucocorticoids administered in vivo impair activation of the immune system, increase pathological effects of disease and increase mortality to viral infections [4].
It has been known for more than 40 years that an immune response with elevated levels of circulating cytokines and activated immune cells can stimulate the HPA axis [5]. These observations were later confirmed in studies utilizing intravenous, intraperitoneal and intracerebroventricular cytokine studies [6-11]. ACTH stimulation will also increase the serum levels of adrenal hormones other than cortisol such as dehydroepiandrosterone and androstenedione [12][13]. These effects can be seen in patients with rheumatoid arthritis or who are undergoing surgery [14][15]. These reactions may include stimulation of peripheral sensory nerves [16][15]. Thus, it is not only cortisol but there are other adrenal steroids which are secreted after a short-term inflammatory stimulus. In this context, it has been demonstrated that high doses of IL-6 stimulate adrenocortical cells in vitro (92).

Chronic inflammatory diseases, however, are associated with elevated levels of cortisol and decreased levels of DHEA while, as described above, acute elevations in inflammatory and immune cytokines increase DHEA and cortisol [17][18]. In long-term inflammatory disease there is a decreased responsiveness of the HPA axis during extended periods of inflammation where IL-6 or TNF serum levels are elevated [10]. Cortisol levels act rapidly on hypothalamic neurons to stop CRH releasing hormone [15]. Ultimately, longer-term elevated proinflammatory cytokines influence adrenal steroid hormone levels [19][20]. In addition, it was observed that there exists an age-related increase of serum IL-6 in healthy female and male subjects associating aging with reduced HPA responsiveness [21].
Dehydroepiandrosterone (DHEA)

Dehydroepiandrosterone (DHEA) and its sulfated derivative (DHEA-S) are the most abundant circulating steroid hormones in humans [22][23]. Despite large amounts of study, the molecular, cellular, physiological mechanisms of action remain elusive. DHEA is an adrenal cortical steroid classified as a neurosteroid which is present in high concentrations during gestation, and age related development [24]. DHEA has been classified as a neurosteroid because it can be synthesized de novo in the brain [25]. Additionally, astrocytes and neurons will convert pregnenolone to DHEA in the brain [26].

The levels of DHEA peak during reproductive years and then decline dramatically with age [27]. DHEA is found to be regulated by the HPA in acute and chronic inflammatory situations. DHEA, because of its biochemical, physiological, pharmacological, toxicological and clinical effects sparked intense scientific interest and a multitude of research efforts were undertaken and are currently ongoing to delineate the beneficial biological effects and action of DHEA.

DHEA has been intensely studied for its effects in treating cancer [28], viral and bacterial infection [29][30][350], auto-immune disease [31], arthritis [32], cardiovascular disease [33], control of body weight [34], stress [30][35], asbestosis [36], neural effects [37], diabetes (metabolic disease) [38], enzyme regulation [39], anti-oxidant effects [40], protection from ischemic/reperfusion [41], effects in bone metabolism [42], and an anti-glucocorticoid effect in promoting immune cell survival and proliferation [43]. DHEA is the parent hormone to its more active metabolites, however, the multitude and significance of the results obtained from DHEA are worth noting here for utility and perspective.
Anticancer effects of DHEA

The effects of anti-carcinogenic effects of DHEA have been well studied. DHEA was shown to produce a decline in tumor burden in castrated mice with human prostate tumors that were propagated in nude mice [28]. Subsequently, fluorinated DHEA was studied because did not convert into estrogen and testosterone [44]. It was shown that 16 alpha-fluoro-5-androsten-17-one, a non-androgenic DHEA analogue, significantly decreased the incidence of small intestinal tumors however, an increased dose was not as effective [45]. DHEA and 16 alpha-fluoro-5-androsten-17-one reduced tumor initiation, tumor promoter-induced epidermal hyperplasia and promotion of papillomas in the two stage skin tumorigenesis model [46]. Together these observations suggested that DHEA alone has some anticancer effects although some activity may be attributed to its metabolites.

DHEA was found to reduce the incidence and multiplicity during both phases of mammary cancer and incidence was most affected by DHEA together with 4-HPR(n-(4-hydroxyphenyl) retinamide) [47]. DHEA also delayed tumor development in p53 knockout mice [48]. DHEA also was associated with increased bone density and lowered serum triglyceride levels in rats with DMBA-induced mammary cancer [49]. Many more studies have associated DHEA with anti-cancer effects [50-53] to name a few. It must be noted that Hamilton et al. paradoxically found that DHEA actually increased colon tumor incidence when male F344 mice were fed 0.5% DHEA for 7 weeks beginning one day after the administration of azoxymethane [54].
Immune effects and DHEA

Multiple reports of the anti-viral effects of DHEA were reported in the 1990's. It was reported that DHEA administration to C57BL/6 mice infected with Murine retrovirus opposed retrovirus induced oxidative damage and loss of immune cytokines (IL-2, IFNγ) [55]. In these studies, DHEA opposed IL-6 and TNFα production by T helper 2 cells (TH2) and DHEA-S was found to oppose retrovirus induced T cell reduction in old mice. Subsequently, the effects of DHEA administration on lethal viral infections was studied and it was found that a single subcutaneous dose of DHEA was able to upregulate the immune response and provide protection against of lethal infective titers of Herpes virus type 2 encephalitis or systemic Coxsackievirus B4 infection [56][350]. Subsequent studies demonstrated that DHEA/DHEA-S could provide significant benefits against immunodeficiency viruses by decreasing virus replication in vitro and provided protection against retrovirus-induced lipid peroxidation in the heart in vivo [57-59].

The ability of DHEA to influence lipopolysaccharide (LPS)-induced TNFα and endotoxic shock was studied and the results demonstrated that DHEA significantly reduced the levels of LPS-induced TNFα and serum corticosterone levels which shows that DHEA is also influencing the stress response. The ability of DHEA to influence resuscitative trauma and the post-traumatic effect of LPS on the systemic inflammatory response was examined [60]. The results demonstrated that DHEA was insufficient in a pig model to protect against progressive shock and pulmonary failure at 4, 10 and 20 mg/kg doses administered at 1, 24, 48 and 72 hours after trauma.
Anti-autoimmune effects and DHEA

It was discovered that 200mg/day of DHEA decreased systemic lupus erythematosus (SLE) activity [61]. Later clinical studies by this same group demonstrated that DHEA treatment alleviated SLE symptoms and systemic manifestations [31]. Subsequently, cytokine studies demonstrated that DHEA levels were found to be low in SLE patients and that DHEA upregulated IL-2 production of normal T cells and reversed the effects of SLE in these patients [62][63]. These results prompted suggestions that low levels of DHEA may be responsible for defects in IL-2 synthesis [62]. Additionally, it was reported that DHEA also reduced the incidence and severity of collagen-induced arthritis [32]. DHEA-S was subsequently shown to be increased after TNFα inhibition in rheumatoid arthritis patients [64]. Together, these results strongly suggest that low DHEA in conjunction with TNFα levels underlie these diseases.

Neural effects and DHEA

It was discovered that DHEA (500mg) given orally to normal subjects induced significant increases in rapid eye movement (REM) sleep during the first 2 hour sleep period [65]. These studies demonstrated that DHEA had different effects at different stages of sleep suggesting that DHEA had a mixed GABA-A agonist/antagonist response [65]. DHEA, administered intracerebroventricularly, was found to improve memory and it was postulated that DHEA converged as a transcription facilitator for immediate -memory functional genes[66]. Another group discovered that DHEA administration over 18 months resulted in counteracting the age-induced suppression of CRH mRNA levels in the hypothalamic periventricular nucleus of both sexes [67]. Additionally, it was found that the decline in DHEA levels in aged people may be
related to pathological amyloid precursor protein metabolism[68]. These results strongly
demonstrate a link between the HPA axis, low DHEA and age-related dementia.

**DHEA, stress and the anti-glucocorticoid Response**

In an elegant experiment, it was shown that mice inoculated with West Nile Virus and
subjected to cold stress followed by serial injection with DHEA(10-20 mg/kg) were protected
from viral infection in the blood, brain and lymphoid organs [30] providing additional support
that DHEA is an anti-stress agent. DHEA was also utilized to treat high and low anxiety in rats
and it was reported that DHEA significantly decreased behavioral despair associated with high
anxiety while there was no significant effects noted in rats with low anxiety [35]. DHEA was
also administered to sound stressed male Sprague-Dawley rats and it was noted that DHEA
blocked the stress induced tryptophan hydroxylase activity in the mid-brain and cortex regions of
the brain [69]. In these experiments DHEA was administered in conjunction with estrogen,
progesterone, testosterone and a glucocorticoid agonist and found that estrogen, testosterone and
progesterone had no effect in blocking sound stress induced tryptophan hydroxylase activity
while DHEA alone or in combination with the glucocorticoid inhibitor blocked sound stress.

Tryptophan hydroxylase is an enzyme that is increased by glucocorticoids and limits the
production of 5-hydroxytryptamine the ligand of the serotonin receptors [70]. Furthermore these
receptors are responsible for modulating and releasing multiple neurotransmitters including
GABA [71]. Glucocorticoid activity (RU 28362) did not increase the sound stress-induced
production of tryptophan hydroxylase in the presence of DHEA and it was suggested that DHEA
achieved this activity through antiglucocorticoid action [69]. It was discovered that high dose
DHEA pretreatment antagonized dexamethasone-induced thymic and splenic atrophy at 60 mg/kg while low doses (10^{-7} to 10^{-8} M) did not provide protection [43].

In addition, it was reported that DHEA alone would not block tyrosine aminotransferase (a glucocorticoid induced enzyme), however DHEA in the presence of dexamethasone blocked dexamethasone induced suppression of tyrosine aminotransferase and ornithine decarboxylase in a time and dose dependent fashion demonstrating the DHEA association with glucocorticoid transcribed genes [72][73]. Studies on the effects of DHEA on levels of the glucocorticoid receptor demonstrated that levels of the glucocorticoid receptor were significantly reduced by DHEA [74]. Together, these observations demonstrate that complex interactions are occurring that involve DHEA, the glucocorticoid receptor and influence the transcriptional responses affecting immune responses, cancer and stress.

**DHEA as a precursor to androstene hormones**

The effects of DHEA have commonly been performed in animal models. DHEA administered as a food admixture resulted in female rats obtaining plasma levels of DHEA that were an order higher than that of the male rats [75][76]. Tissue distribution of DHEA was subsequently studied in male and female Sprague-Dawley rats following oral administration and it was observed that there were no main sex differences between male and female rats[74]. DHEA levels rapidly decreased within 24 hours and was found to be primarily retained mainly in the liver, kidney, pituitary and bone marrow [74]. The presence of radioactive DHEA was observed in the pituitary and bone marrow even when levels of radioactivity were significantly decreased [74].
A significant difference in DHEA excretion was found to exist between males and females. Male rats excrete DHEA in the faeces while female rats excrete DHEA metabolites in the urine [74]. Both sexes excrete the DHEA metabolites in the first 24 hours after ingestion [74]. While sex differences in excretion exist between serum, urine, bile, liver and faeces, the metabolite that was found to be present in the greatest concentration was Δ5-androstene-3β, 17β-diol (17β-AED) in female rats and more polar DHEA metabolites including Δ5-androstene-3β, 17β-diol (17β-AED) in males [74]. The presence of polar metabolites to dominate demonstrated that these metabolites may have significance. It was observed that DHEA is only found in trace amounts after administration and excreted polar metabolites with a hydroxyl group in the 7 position were present. Importantly, 7-hydroxylated steroids had been observed to have an important role in the immune stimulation of mice [77][78]. Thus, in addition to DHEA, considerable attention has been given to the study of 17β-AED, and Δ5-androstene-3β, 17β-triol (17β-AET).

Δ5-androstene-3β, 17β-diol (17β-AED) and Δ5-androstene 3β, 17β-diol (17β-AET) Metabolism

Δ5-androstene-3β, 17β-diol (17β-AED) is one of the primary metabolites derived from DHEA. 17β-AED is a derivative of DHEA which results from conversion of the keto group to a hydroxyl group at the 17 position [74]. 17β-AED is hydroxylated at the 7 position before Δ5-androstene-3β, 17β-triol (17β-AET) can be formed [79]. The biological activity of DHEA, 17β-AED, 17β-AET has been demonstrated to occur through the subcutaneous route [80][81]. The skin, in turn, has been shown to contain all the necessary enzymes to convert DHEA to 17β-AED and 17β-AET [82-85]. 17β-AED metabolism has been evaluated in men and women and it has been found circulating in the serum [86][87]. Metabolism of Δ5-androstene-3β, 17β-diol (17β-
AED) was evaluated and found to be comparable in men and women and was converted into DHEA, 17β-AED sulfate and DHEA-S [87]. An important observation is that DHEA can be transformed into 17β-AED and conversely, 17β-AED can be transformed back to DHEA [74][87]. This indicates that some interconversion can occur which may be related to DHEA or 17β-AED functionality where this interconversion occurs.

\[ \text{DHEA, } \Delta^5\text{-androstene-3\beta, 17\beta-diol (17\beta-AED), } \Delta^5\text{-androstene-3\beta, 7\beta, 17\beta-triol (17\beta-AET),} \]

immunity and antiglucocorticoid activity

\( \Delta5\text{-androstene-3\beta, 17\beta-diol (17\beta-AED)} \) is a DHEA metabolite that possesses considerable ability to upregulate immunity in a generalized and non-specific fashion [88]. It was determined that DHEA was not the active agent that induced the host immune response [80]. Consequently, it was shown that 17β-AED, at 1/3 the dose of DHEA, is at least 100 times more effective than DHEA at increasing host immunity needed to protect against many different types of infection including viral, bacterial, parasitic and non-infectious agents [90]. 17β-AET possesses 10,000 times the efficacy of inducing the host immune response than is DHEA [89].

17β-AED was tested in vivo to determine the most effective route of administration and effective dosage [91]. It was shown that 17β-AED, by the cutaneous route, would protect against lethal infection at 20, 80 or 160 mg/kg doses, however, it was determined that only 320 mg/kg 17β-AED only in the presence of an antigen would induce spleen and thymus proliferation [91]. This is in contrast to DHEA which did not induce spleen or thymus proliferation [91]. In these studies, heart destruction by coxsackie virus-induced cytotoxic lymphocytic activity was noted to be absent when 17β-AED was present thus demonstrating that host cytotoxic lymphocyte activity was inhibited even in the presence of the infecting virus indicating that a non-
glucocorticoid, anti-inflammatory action could be achieved through host immune modulation [91].

DHEA and 17β-AED were studied for their ability to protect mice against lethal bacterial infection and LPS toxicity [88]. 17β-AED was shown to have no effect on TNFα levels that was induced to an in vivo LPS challenge in mice [88]. In opposition and similarly to glucocorticoids, DHEA inhibited TNFα concentration in these studies [29][88]. Furthermore, in vitro experiments with RAW 264.7 macrophages demonstrated the same effects that were seen in vivo[88].

In addition to DHEA and 17β-AED, Δ5-androstene-3β, 7β, 17β-triol (17β-AET ) was synthesized and found to be even more potent than 17β-AED in upregulating the immune response in vivo [81]. 17β-AET was also found to possess anti-glucocorticoid activity [92]. Mitogen treated spleen cells were exposed to 17β-AET, 17β-AED and DHEA and it was reported that 17β-AET increased IL-2 and IL-3 while 17β-AED had no effect and DHEA decreased the levels of these two cytokines that are suppressed by glucocorticoids [92]. Because 17β-AED and 17β-AET were effective in upregulating host immunity in vivo and displayed anti-glucocorticoid activity that was different from DHEA and each other, further studies were pursued to further examine the anti-glucocorticoid effects on immune mediating macrophages and lymphocytes.

Studies were performed with DHEA and 17β-AED on the ability of these androstene hormones to counteract the action of glucocorticoids on macrophages and lymphocytes in vitro [93][78]. Glucocorticoids have been reported to downregulate TNFα, IL-1, and IL-6 [94-96]. In LPS stimulated RAW264.7 macrophages, 17β-AET increased IL-1 and TNFα, 17β-AED did not affect IL-1 or TNFα, while DHEA decreased both IL-1 and TNFα [97]. Strikingly, all three androstene hormones decreased IL-6 to the same level as hydrocortisone in these cells [93]. In
mitogen stimulated lymphocytes a similar pattern was noted for IL-2 and IL-3 suppression by hydrocortisone where 17β-AET in the presence of hydrocortisone significantly increased IL-2 and IL-3, 17β-AED increased IL-2 and IL-3 at high concentrations to levels lower than 17β-AET, while DHEA maintained levels of IL-2 and IL-3 at the same level as the hydrocortisone control [78]. Together, these results demonstrate that immune mediating macrophages and lymphocytes respond differentially to DHEA, 17β-AED and 17β-AET and display an increasing anti-glucocorticoid and immune functionality from DHEA to 17β-AED to 17β-AET.

Together, these results demonstrate that DHEA, 17β-AED and 17β-AET differentially regulate immune responses in vivo and in vitro and also possess differential activity with glucocorticoids. Because DHEA, 17β-AED and 17β-AET possess different anti-glucocorticoid activity in vivo and in vitro then open questions are 1) Do they each interact with the glucocorticoid receptor differently at the level of the glucocorticoid receptor? and 2) Do they each interact with the glucocorticoid receptor at all?

Transcriptional Effects of 17β-AED and 17β-AET

DHEA, 17β-AED and 17β-AET possess different anti-glucocorticoid activity in vivo and in vitro. Glucocorticoids and gamma irradiation both induce immune injury. Glucocorticoids cause destruction of lymphoid cells and alters RNA synthesis [4]. Gamma irradiation damages genetic material in all blood components and mediates destruction of lymphocytes[98]. Thus, and 17β-AET were evaluated on their ability to counteract the damaging effects of whole body ionizing radiation [99-101]. It was discovered that not only did 17β-AED and 17β-AET protect against a lethal radiation dose of 8Gy(800 rad) at subcutaneously administered concentrations of 320 mg/kg for 17β-AED and 30 mg/kg for 17β-AET but also increased immune protection
against Coxsackie B4 virus as they did with unirradiated animals [81]. Together, these results demonstrate that both 17β-AED and 17β-AET produce survival against the damaging and destructive effects of lethal irradiation and at the same time restore the immune effects.

Ultimately, it was discovered that 17β-AED stimulated protection and accelerated multilineage blood cell recovery and elevated bone marrow (BM) cellularity [101]. Spleen colony-forming unit assays showed that combined treatment with 5-AED plus thrombopoietin resulted in a 3 to 4 fold increase as opposed to 5-AED and TPO alone [101]. In opposition to thrombopoietin, 17β-AED demonstrated protection and survival of bone marrow progenitors [100] [101]. These studies identified transcription factors and cytokines present in the response to irradiation 17β-AED was associated with elevation of GM-CSF, IL-2, IL-3, IL-6 and IL-10 in the spleen before irradiation and GM-CSF, IL-2 in bone marrow. Post-irradiation G-CSF, GM-CSF, Interferon gamma, thrombopoietin, IL-2, IL-3, IL-6, IL-10 and IL-12 were all elevated in spleen and GM-CSF, interferon gamma, thrombopoietin, IL-3 and IL-10 in bone marrow [102]. Expression of G-CSF was associated with the master transcriptional regulator: NFkB1 in response to irradiation [100][103]. Because increased levels of CDKN1A, BCL2, BAX and DDB1 were observed, 17β-AED was associated with cellular programs of DNA damage prevention, cell cycle progression and apoptosis. These results demonstrate that 17β-AED, through a program distinct from thrombopoietin, will direct the expression of cytokines in response to irradiation and suggest that these cytokines are the result of a transcriptional program inclusive of NFkB1.

17β-AED and 17β-AET possess different anti-glucocorticoid activity in vivo and in vitro and demonstrated ability to direct transcriptional programs that result in survival in response to stress. 17β-AED and 17β-AET were subsequently tested for their ability to improve otherwise
depressed cardiac function and cytokines after trauma hemorrhage [104]. It was observed that 17β-AED significantly improved blood flow in the liver, brain, kidney, pancreas, spleen and adrenal glands, significantly reduced IL-6, and raised nitrates/nitrites [104]. It was also reported that 17β-AED improved hepatic portal function through decreasing endothelin-1 and increasing eNOS [105].

Subsequently, it was reported that peroxisome proliferator activated-receptor gamma (PPARγ) was at least, in part, responsible for the beneficial decrease in IL-6 and iNOS [106]. 17β-AET also was found to provide protection against trauma hemorrhage and it was reported that 17β-AET decreased IL-6 while increasing IL-2 and IFNγ in the spleen [107][108]. Together these observations demonstrate that 17β-AED and 17β-AET induction of transcriptional programs resulting in survival that are at least, in part, mediated by transcriptional master regulators (PPARγ).

17β-AED possesses anti-glucocorticoid activity in vivo and in vitro. Stress and the glucocorticoid receptor (GCR) are known to delay wound healing [109-112]. Blockade of the GCR increased IL-1β and keratinocyte growth factor 1 KGF-1 levels in these studies and increased wound cellularity and returned wound healing to normal [109]. Similarly, 17β-AED reversed glucocorticoid suppression of IL-1β and PDGF [112]. 17β-AED thus appears to also affect gene expression patterns in cutaneous wounds through the GCR transcriptional regulator.

DHEA, 17β-AED, 17β-AET and the estrogen/androgen receptors

Estrogen and Androgen are master transcription regulators that are members of the nuclear receptor superfamily [113]. Cutaneous wound healing and neuroprotective activity has generally been attributed to estrogen derived from DHEA [26][114-116]. Subsequently, it has
been demonstrated that cutaneous wound healing is associated with the action of the estrogen receptor beta (ERβ) and that androgen opposed the action of ERβ [117]. 17β-AED has inherent androgenic and estrogenic properties [118-120]. Furthermore, studies of trauma-hemorrhage have associated increased NFκB and activator protein-1 (AP-1) with increased IL-6 and TNFα [121]. Normalization of all these proteins was obtained by 17β-estradiol and was associated with PPARγ [121]. These observations have led some to hypothesize that androstene hormones may function similarly to estrogen [122].

It has been known for some time that 17β-AED possesses both androgenic and estrogenic activity [123]. Several studies demonstrate the activation of the estrogen and androgen receptor by C19 steroids with resultant "estrogenic effects" (i.e. gain in uterine weight) "androgenic effects" (i.e. hirsutism), or proliferation of human mammary cancer cells [124-130]. A "google scholar" search of "estrogen and proliferation" returned 685,000 results while the same search with androgen yielded 163,000 results. Clearly, androgen and estrogen are associated with proliferation. It was noted that 17β-AED was present in high concentrations in some proliferative diseases, mainly breast cancer [131] and prostate cancer [132]. Some studies reported that androgenic metabolites may compete with 17β-estradiol and translocated the estrogen receptor in the uterus and mammary tumors [133-137]. Thus, it was thought that 17β-AED, either itself or as a metabolite, was responsible for cellular proliferation through an interaction with either the androgen or estrogen receptor.

It was found that in mammary cancer, estrogen stimulated MCF-1, ZR-75, T47-D and EFM-19 cells [138-141]. It was reported that high concentrations of androgen enhanced growth of EFM-19 and MCF-7 [141][142] cells while physiological concentrations of androgen were inhibitory in ZR-75-1 and MFM-223 cells[143][144]. Estrogen and androgen receptors were
present and found in different concentrations in all of these cell lines [144]. Subsequently, 17β-AED was tested on MFM-223 (high androgen receptors and low estrogen receptors) and MCF-7 (low androgen receptors and high estrogen receptors) breast cancer cells [145]. 17β-AED was found to be inhibitory in the MFM-223 cells and growth promoting in MCF-7 cells [145]. These studies demonstrated that it took 3 orders of magnitude greater concentration of 17β-AED to cause the proliferative effect in conjunction with the estrogen receptor while the inhibitory effect of 17β-AED on MFM-223 cells was only partially reversed through cyproterone acetate [145].

It has been shown through structural modeling of the estrogen receptor alpha binding pocket that this receptor can accommodate many molecules including 17β-AED [146]. Additionally, it is mentioned that Δ5-steroids, including 17β-AED, may be ancestral ligands of the estrogen receptor [146]. Interestingly, because the estrogen receptor is believed to have undergone convergent evolution this may imply a different functionality for 17β-AED [147]. These results demonstrate that indeed 17β-AED may compete with 17β-estradiol and affect its structure and function.

These results demonstrate that 17β-AED interacts differently than either estrogen or testosterone and that the biological effects are dictated by the composition of the cells. Since, there can be opposing effects within the same cell type; it is unclear the effects of DHEA are due strictly to the estrogen and androgen receptors. Recently, it was shown that 17β-AED is anti-inflammatory in a model of experimental autoimmune encephalitis [148]. It was shown that 17β-AED and not 17β-estradiol inhibited lipopolysaccharide induced IL-6 through a mechanism that involved 17β-AED binding the estrogen receptor beta along with the recruitment of CtBP to tether and suppress the activity of cFos [148]. This suppressive action of 17β-AED is clearly different than the actions of 17β-estradiol on the estrogen receptor.
17β-AED was shown to mechanistically interact with cFos and influence IL-6 differently than 17β-estradiol [148]. These observations further implicate the 17β-AED influence of gene expression in the presence of inflammatory stimuli through another master transcriptional regulator (cFos), in addition to those already discussed (NFkB and PPAR). Thus, the transcription factors that have been studied and reported for DHEA and 17β-AED so far resemble the same transcription factors reported for 17β-estradiol. The biological actions and mechanistic effects, however, are clearly different. Together, the differences in the immune regulation and in anti-glucocorticoid activity attributed to DHEA, 17β-AED and 17β-AET indicate that these receptors may have different interactions at the level of the ER and AR receptors.

\[\Delta^5\text{-androstene-3\beta, 17\alpha-diol (17\alpha\text{-AED)}}\]

17β-AED and 17α-AED are chemically identical and the only difference is the position of the hydroxyl group at the 17-position [149]. 17β-AED is derived from DHEA. 17α-AED is another C19 Δ5-steroid that, like 17β-AED is produced in the testes[150][151]. 17α-AED is found in the amniotic fluid and fetal-placental circulation during pregnancy with the ratio of 17α-AED to 17β-AED of 9:1 in favor of 17α-AED. In adulthood the ratio is 2:1 in favor of 17β-AED [150][151]. 17α-AED and 17β-AED have been associated with epitestosterone production in the human testes [152]. Additionally, while it has not been confirmed, 17β-AED and epitestosterone have been shown to be formed in the human ovarian follicular fluid [153]. Low levels of 17α-AED have been associated with toxemia, diabetes and placental insufficiency [152].

17α-AED was originally tested for biological activity on myeloid oncogenic cell lines [154]. It was reported that 17α-AED had an anti-proliferative effect on murine macrophage 264.7
macrophages, murine p388D1 lymphoid neoplasm cells, human promyelocytic leukemia (HL-60) cells demonstrating an anti-proliferative effect across three different types of myeloid cell types [154]. Additionally, 17α-AED at 50 nM was shown to produce irreversible apoptosis shown by electron microscopy in 2 (HL-60 and Raw 364.7) of the 3 cell lines while 17β-AED, the epimer of 17α-AED, at the same concentration did not produce cell death.

HL-60 cells were treated with either 17β-AED or 17α-AED and it was discovered that 17β-AED at all concentrations promoted incorporation of [3H] thymidine and thus demonstrated DNA synthesis as opposed to 17α-AED which at concentrations at or above 12.5 nM didn't promote [3H] thymidine uptake [154]. The [3H] thymidine uptake test was also performed for Raw 264.7 cells and it was demonstrated that [3H] thymidine uptake was inhibited at 17α-AED concentrations at or above 50 nM and, in this cell line, 17β-AED produced a decrease in the [3H] thymidine uptake at levels at or above 500 nM [154]. In the p388D1 lymphoid cells, 17α-AED produced a significant reduction in [3H] thymidine uptake and produced an anti-proliferative effect at concentrations at or above 12.25 nM while 17β-AED did not decrease DNA synthesis at any concentration [154]. Together, these results show that DNA synthesis correlated with an anti-proliferative effect for 17α-AED and a proliferative effect for 17β-AED in these myeloid cells lines.

Because anti-proliferative effects were demonstrated by 17α-AED on the myeloid cell lines this epimer of 17β-AED was tested for anti-proliferative ability in the ZR75-1 (estrogen positive) and MDA-MB231 (estrogen negative) human mammary cancer cell lines [155]. Because the chemically identical epimer, 17β-AED, is produced in primary breast tumors [156] and 17β-AED is known to possess an ability to interact with the estrogen and androgen receptors [119], these tests were performed in the ZR75-1 and MDA-MB231 cell lines.
The results demonstrated that similar to myeloid cells, 17α-AED inhibited proliferation of ZR75-1 and MDA-MB231 cells between the concentration of 12.5 to 50 nM [155]. These studies were repeated with flutamide blocked androgen receptors and no change was noted in the anti-proliferative ability of 17α-AED on both cell lines [155]. 17α-AED was tested simultaneously with 17β-estradiol on the ZR75-1 cells and an enhanced anti-proliferative effect was noted [155]. 17β-AED and 17α-AED were tested simultaneously on ZR75-1 cells and a profound decrease in proliferation was noted [155].

It was shown in separate experiments with the estrogen inhibitor, tamoxifen and the androgen inhibitor flutamide that 17β-AED appeared to have anti-proliferative effects on MCF-7 cells (androgen and estrogen receptor positive) through the androgen receptor and proliferative effects through the estrogen receptor. Together, these studies associate 17β-AED with estrogen and androgen activity, which was shown to be dependent on the receptor content and "growth properties" of the cells [119] and that 17α-AED will oppose these activities independent of the androgen and estrogen receptors.

17α-AED produced anti-proliferative effects in different cell lines. DHEA, 17β-AED, 17β-AET and 17α-AED are derived from neural tissue. With 17α-AED producing anti-proliferative, and in most cases cell death, these hormones were tested for cytotoxic ability on T98G Glioblastoma and U937 Lymphoma cells [157]. 17α-AED produced irreversible cell death by autophagy at doses above 15uM (90% inhibition at 25uM) in T98G cells and apoptosis in U937 Lymphoma cells. In contrast, DHEA, 17β-AED and 17β-AET did not produce cell death. 17α-AED was then tested on GBM6, T98G, U87MG, LN-18, LM-Z308 Glioblastoma cells[158]. All cell lines could be induced to enter irreversible cell death by autophagy and at IC₅₀ concentrations between 8 and 25uM [158]. Additionally, it was discovered that 17α-AED
reduced AKT/mTOR signaling and induced autophagy through the induction of ATG5 and beclin-1 in these cell lines. Together, these observations suggested a mechanism that could induce different irreversible programmed death pathways in different cell types.

A hallmark of steroid hormones is that slight changes in hormone structure can lead to significant differences in biological functionality [159]. Due to this characteristic of steroid hormones, 6 androstene hormones with the hydroxyl groups at different positions were synthesized and tested to determine cytotoxic effects on T98G Glioblastoma and U937 Lymphoma cells[160]. It was discovered that the position and orientation of the hydroxyl group at the 17 position in relation to the cycloperhydrophenanthrene steroid ring and not the hydroxyl located at the 3 position was responsible for either autophagy or the apoptotic effect on Glioblastoma and Lymphoma cells respectively [160]. These observations suggest that the hydroxyl at the 17 position impart 17α-AED with the ability to direct different programmed cell death pathways in different cell types and that these effects are through estrogen/androgen independent means. Because 17α-AED and 17β-AED, other than the orientation of the hydroxyl group at the 17 position, are chemically identical, it is possible that 17α-AED could fit in the estrogen and androgen ligand binding pocket. Despite the anti-proliferative activity, it is currently unknown whether 17α-AED interacts with the androgen and estrogen receptors.

Pursuant to these observations, the mechanism of action of 17α-AED was investigated [161]. LN-18, LN-229, U87MG, LN-Z308, U251MG Glioblastoma cells were treated with 17α-AED and members of the unfolded protein response were investigated since prior results implicated ATG5 and beclin-1 suggesting that a class III PI3K/p150 lipase signaling complex was involved [161]. Experiments were performed utilizing these multiple cell lines and T98G Glioblastoma cells transfected with either a double negative mutant PERK or an empty vector.
control [159]. The results demonstrated that 17α-AED activated the double stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) and not interferon-inducible double stranded RNA dependent activator kinase (PKR), the eukaryotic translation initiation factor 2 alpha kinase 4 (GCN2) or the eukaryotic translation initiation factor 2-alpha kinase 1 (HRI) [161]. It was then further shown that phosphorylation of eLF2α occurred downstream of PERK activation which is consistent with translation attenuation [161]. Additionally, it was shown that the X-box binding protein 1 (XBP-1) was not cleaved while CCAAT/enhancer binding protein homologous protein (CHOP), glucose regulated protein of 78 kDa (GP78) were elevated which further suggest a specific mechanism of action [161]. Together, these observations demonstrate that 17α-AED possesses mechanisms of action that are independent of estrogen and androgen receptors.

The unfolded protein response (UPR) typically involve the three endoplasmic reticulum transmembrane receptors: activating transcription factor 6 (ATF6), inositol requiring kinase (IRE1) and double stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) [162]. The UPR performs three functions; adaptation, alarm, and apoptosis [162]. Adaptation is associated with a translational block and expression of chaperones to aid in refolding [162]. If this is unsuccessful then the alarm phase is initiated which is associated initiation of signal transduction events that lead to removal of the translational block, downregulation of survival proteins (B-cell CLL/lymphoma 2 (BCL2)) and after the alarm phase ER stress can activate programmed cell death [162].

While the activation of PERK by 17α-AED could be associated with a UPR, there are a few observations that are different from an ER unfolded protein response. First, the UPR is associated with IRE1 and ATF6 activation [162]. There was no activation of XBP-1 or activation
of either the IRE1 or ATF6 by 17α-AED [161]. The translational block was not released during autophagy induced in the Glioblastoma cells by the 17α-AED thus the translational block accompanied the induction of autophagy. Last, there was an expression of Grp78 which is consistent with the adaptation stage of the UPR. These results are consistent with a direct activation of PERK along with a sustained translational block leading to irreversible programmed cell death. The upregulation of CHOP and the signal transduction modulation implied by AKT/mTOR [158] suggests that there are specific transcriptional events that are associated with the activation of PERK by 17α-AED, however, further knowledge of these events are currently unknown.

**Overview**

The observations presented in this brief introduction of DHEA, 17β-AED, 17β-AET and 17α-AED were intended to demonstrate the observations central to the idea that the androstene hormones exhibit a specific biological functionality that is dependent on their structure and conformation. The androstene hormones have been associated with the medically critical, cell-specific responses of repair and survival or death. The wide range of beneficial effects demonstrated by the androstene hormones are associated with receptors and cytokines that are transcriptionally regulated by master transcription regulators that include NFkB, cFOS) and members of the nuclear receptor superfamily (AR, ERα, ERβ, GR).

This dissertation was based on three hypotheses. In Chapter 1, the operating hypothesis is: Androstene Hormones, based on their putative ability to interact with the androgen and estrogen receptors and yet promote differential anti-glucocorticoid activity, will demonstrate differential activation of the human ERα, ERβ, AR and GR, at the level of the receptor and will
demonstrate decreased androgenicity or estrogenicity compared to 17β-estradiol and testosterone. In Chapter 2, because 17α-AED but not 17β-AED induces irreversible autophagy in Glioblastoma cells and that induction is associated with the chemical structure and conformation (specifically the hydroxyl group at the 17 position) of 17β-AED and 17α-AED then the operating hypothesis is: 17β-AED and 17α-AED will be associated with unique transcriptional regulation in Glioblastoma cells. Finally, in chapter 3, because 17β-AED regulates host resistance through skin associated immunity and possesses the ability to regulate transcription we tested the hypothesis that transcriptional regulators modulated by 17β-AED would influence genes which are significantly active in the pressure ulcer wound edges in comparison to the normal skin.
CHAPTER 2: Structural Stereochemistry of Androstene Hormones Determines Interactions with Human Androgen, Estrogen, and Glucocorticoid Receptors

Introduction

DHEA, an androstene hormone, has been shown to possess a wide range of beneficial biological effects mainly attributed to immune system modulation [163]. DHEA, is metabolized into more active metabolites i.e., 17β-AED, 17β-AET as well as testosterone and estradiol [163][164]. 17β-AED and 17β-AET have been reported to prevent the morbidity and mortality of otherwise lethal infections [89][165] potentiate lymphocyte activation and counteract the immune suppressive action of hydrocortisone [166][167] thus leading to beneficial effects in diverse human diseases including resistance to infection, neuroprotection, wound healing, diabetes, hepatic injury, cardiovascular disease and cancer [168-170].

17α-AED mediates autophagy of glial and breast cancers and apoptosis of myeloid tumor cells [154][160][171]. 17β-AED and 17α-AED naturally exist in epimeric forms based on whether the hydroxyl group is above (β) or below (α) the Δ5 cycloperhydrophenanthrene ring. Addition of a hydroxyl group at the C7 position to 17β-AED results in the formation of Δ5-androstene-3β, 7β, 17β-triol (17β-AET). The biological activities of 17α-AED, 17β-AED and 17β-AET have exhibited a structure-activity relationship that depends on the orientation and location of the hydroxyl groups [172]. Androstene hormones (AH) have been shown to promulgate their biological effects in many different animal models including mice, rats,
monkeys and some specific human tissues. Reports have associated the mechanism of action of androstene hormone metabolites with androgen, estrogen and glucocorticoid receptor activity [112][118][145]. Adrenal hormones have been shown to activate both androgen and estrogen constructs. In this regard, it has been documented that 17β-AED can activate the AR receptor in prostate tissue in the presence of commonly used anti-androgens [173]. Inhibitors of both the androgen receptor and the estrogen receptors demonstrated that AR and ERβ receptors combine to affect gene transcription [174]. Additionally, 17β-AED was recently shown to be a part of an anti-inflammatory mechanism that utilizes the ERβ [148]. 17β-AED and 17β-AET have been documented in vitro and in vivo to oppose the action of hydrocortisone indicating that there may be crosstalk with the GR receptor [78][91][92].

DHEA has been shown to possess weak androgenicity and estrogenicity [175]. Because 17α-AED, 17β-AED and 17β-AET are more potent metabolites of DHEA that exhibit strong biological activity that could be attributed to androgenic, estrogenic or anti-glucocorticoid activity in vivo and in vitro it was advantageous to identify whether or not this is directly mediated by the human ER, AR and GR receptors at the cellular level. Additionally, androstenediol has been modeled as a chemical with a 3β-hydroxy and a saturated A ring which can act as an estrogen [176]. Consequently, we compared DHEA, 17α-AED, 17β-AED and 17β-AET in the Indigo Biosciences nuclear receptor assay system for their ability to activate the human AR, ER and GR receptors and determine the relative androgenicity and estrogenicity of these androstene hormone derivatives.
Methods

Nuclear Receptor Transactivation Assays

Nuclear receptor transactivation assays were obtained from Indigo Biosciences (State College, PA, USA) and were utilized to assess activation of human AR, ERβ and ERα receptors. Briefly, stocks of the compounds tested were prepared and diluted in medium provided by the manufacturer. Cell medium was tested for hormone activity by mass spectrometry. Frozen reporter cells provided in the assay kit were thawed and compound dilutions were added immediately. Cells were incubated for 24 hours and the activation response was measured on a luminometer (Perkin-Elmer, MA, USA). The cells consisted of non-human mammalian cells engineered by Indigo Biosciences to provide constitutive high-level expression of full length, unmodified human androgen Receptor (NR3C4), human estrogen receptor 1 (NR3A1), human estrogen receptor 2 (NR3A2) and of full length, human glucocorticoid Receptor (NR3C1).

The non-human mammalian reporter cells included a luciferase reporter gene functionally linked to a human nuclear receptor-responsive promoter. The cells are engineered so that only interactions with the human receptor will induce luciferase expression in the treated reporter cells to quantitate nuclear receptor activation. Positive control ligand performance was measured by the manufacturer and provided in the technical manuals thus allowing accurate comparison for assay performance. Additionally the control ligands of the receptors (testosterone, 17β-estradiol, dexamethasone) were tested on the same test plates (n=3 to allow statistical analysis) with the androstene hormones and controls.
Preparation of Stock Hormone Solutions

Stock solutions of 17β-AED, 17α-AED, Testosterone (Sigma-Aldrich, St. Louis, MO, USA) were prepared by dissolving the compounds into 100% ethanol to a final 50 mM stock solution concentration; 17β-AET was also a stock solution of 50 mM but was dissolved into 1:1 DMSO/Ethanol because of solubility issues. Cyproterone Acetate (Sigma-Aldrich) was diluted with 100% ethanol and used at a 10 uM concentration. All stock solutions were diluted to final concentrations using the dilution fluid provided in each kit. All tests were performed with negative controls on the same plate and contained media alone and media containing the same amount of ethanol utilized in the stock solutions. All assay control results were in accordance with the stated technical performance specifications.

Mass Spectrometry

LC-MS/MS analysis of the steroid hormones were carried out using a Shimadzu Nexera UPLC device coupled (Shimadzu Corp, Kyoto, Japan) to ABSciex (Foster City, CA) 5500 Hybrid Triple Quadrupole Linear Ion Trap Mass Spectrometer operating in multiple reaction monitoring mode. Nitrogen produced by a high-purity nitrogen generator (PEAK Scientific Instruments Ltd, Chicago, Ill) was used as curtain, nebulizer and collision gases. Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3 Ionization of the analytes were carried out using an APCI source. Multiple MRM transitions were selected for each analyte to eliminate ambiguity in analyte identification. For all steroids other than AED, 25 microliters of the media was directly injected onto a 2.1 x 50mm 2.6 μm C18 Reverse Phase column (Phenomenex) and was separated via a linear gradient of water:methanol 98:2 (Solvent A) to methanol:water 70:30 (Solvent B). Both solvents contained 5mM ammonium formate with 1% formic acid. Separation of 17α-AED
and 17β-AED was carried out using 2.1 x 150 mm 2.6μm C18 Reverse Phase column (Phenomenex) and was separated using same Solvent A as above and 98:2 methanol: water with 5mM ammonium formate and 1% formic acid as Solvent B. Steroid hormones were detected using precursor-product MRM pairs are as follows; DHEA (271-213, 271-197), Androstenetriol (307-158, 307-254), Androstenediol (291-95, 291-159, 291-255), Testosterone (289-97, 289-109), Androstenedione (287-97, 287-109), 17β-estradiol (273-107, 273-135, 273-77). Where there were multiple transitions, the dominant peak was used in the analysis. All analytes demonstrated a minimal limit of detection of at least 0.6 nM.

Cellular Uptake of Androstene Hormones and Normalization of Transactivation Assay Results

It was relevant to determine the relative uptake of each androstene hormone in the Indigo Assay System. Cells and cell medium utilized in the assays were provided by the manufacturer (Indigo Biosciences). The following components were found to be below the limit of detection in the cells and cell medium: DHEA, 17β-estradiol, androstenedione, testosterone, 17α-AED, 17β-AED and 17β-AET. LCMS data was utilized as a ratio between the background subtracted signal (area under the curve of the LCMS trace) at time zero and 24 hours for each analyte investigated. The ratios were then utilized to create normalization factors for the cellular uptake of 17α-AED, 17β-AED and 17β-AET as compared to DHEA. The normalization factors were: DHEA: 1, 17α-AED: 1.71, 17β-AED: 1.85, 17β-AET: 2.40, respectively. Background activity due to cells, media and vehicle were subtracted before the data were normalized.
Metabolism of Androstene Hormones

The cell medium was analyzed before and after incubation with assay cells. Mass Spectroscopy was utilized to detect androstenedione, 17β-AED, 17α-AED and 17β-AET, testosterone and 17β-estradiol that were expressed in the medium. Approximately 9% of DHEA was metabolized to 17β-AED after a 24 hour incubation with assay cells. No other DHEA metabolites were detected. Mass spectroscopy did not detect any metabolites of the other androstene hormones in the media after a 24 hr incubation period. The Mass Spectrometry data show that DHEA was minimally metabolized to 17β-AED in this cell construct, but not to testosterone or 17β-estradiol (Methods 2.3.2). 17β-AED, 17β-AET and 17α-AED were not metabolized.

Statistical Analysis

All statistical analyses were performed using SigmaPlot version 12(SSI, San Jose, CA, USA). Hormone EC_{50} level and the estrogen receptor alpha activation statistical analyses were performed with a student's t test while all other hormone activation statistical comparisons were performed with a one-way ANOVA. p value levels < 0.05 were considered significant. Statistics on test groups were done before normalization to cellular uptake and were performed between test groups and controls.

Results and Discussion

Androstene hormone structures

The hormones that were used in this study are listed in Figure 1. The structures demonstrate the similarities and unique characteristics of each androstene hormone. The main differences are
the orientation of the hydroxyl group at position C17 for 17α-AED and 17β-AED, the orientation and position of the hydroxyl group at position C7 for 17β-AET, and the ketone group at position 17 for DHEA. 17α-AED and 17β-AED are chemically identical except for the placement of the

**Figure 1. The structures of the Androstene Hormones**

The androstene hormones are shown with the Δ5 cycloperhydrophenanthrene ring. All steroids have a C3 hydroxyl group in the (β) beta position. The C7 hydroxyl group of Androstenetriol is in the β-position. The C17 hydroxyl of Androstenediol epimers are in either the (α) alpha or (β) beta position.
hydroxyl group in relation to (above or below) the $\Delta^5$ cycloperhydrophenanthrene ring. All adrenal hormones in this study, with the exception of DHEA, possess hydroxyl groups in the C3 and C17 position with 17$\alpha$-AED having the C17 hydroxyl group in the ($\alpha$) position. This position at C17 results in remarkable biological actions [154][160] while the hydroxyl group at C3 was shown not to influence the biological activity [177].

**Androstene Hormone Activation of the Human AR receptor**

The data demonstrate that both the orientation of the hydroxyl at position C17 and the addition of the hydroxyl at position C7 affected the ability of 17$\beta$-AED, 17$\beta$-AET and 17$\alpha$-AED to activate the human AR construct (Figure 2). The AR receptor construct contains a luciferase reporter gene that is functionally linked to an AR responsive promoter. The luciferase reading is utilized as a surrogate measure for AR binding. The EC$_{50}$ was calculated for testosterone as the 50% activation point. EC$_{50}$ values for the androstene hormones and testosterone were calculated (Table 1.) and compared utilizing the ratio of the androstene hormone EC$_{50}$ to testosterone EC$_{50}$ (Relative Androgenicity). All of the androstene hormones tested showed a significant (p<0.001) reduced androgenicity when compared to testosterone (Table 1). 17$\beta$-AED had only 1/5th the ability of testosterone to activate the androgen receptor. Changing the orientation of the C17 hydroxyl group on 17$\alpha$-AED resulted in a further reduction to 1/60th the activity as compared to testosterone. Addition of the hydroxyl group to the C7 position further reduced the ability to activate the human AR construct to 1/1326th as that of testosterone. The androstene hormone activation of the human AR was rank ordered based on strength of activation. The rank order was: 17$\beta$-AED>>17$\alpha$-AED>>>17$\beta$-AET (Figure 1). DHEA binding to the AR receptor was excluded from these experiments since its androgenicity has been reported previously [175].
Figure 2. Androstene Hormone Activation of the Human Androgen Receptor

Reporter cells were treated with 17α-AED or 17β-AED or 17β-AET (n=3) at each concentration, incubated for 24 hours and then assayed for luciferase activity. Androstene hormone activity was normalized to cellular uptake (Methods). Error Bars, ± 1SD. Statistical significance, p < 0.001 versus androstene hormone metabolites in the same concentration group (¤), p<0.001 versus androstene hormone metabolites in the same concentration group (*).
Table 1. Relative Androgenicity of Androstene Hormones

<table>
<thead>
<tr>
<th>Hormone</th>
<th>EC$_{50}$(nM)</th>
<th>Androgenicity</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>0.35</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$\Delta^5$-androstene-3(\beta), 17(\beta)-diol</td>
<td>1.8</td>
<td>1/5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$\Delta^5$-androstene-3(\beta), 17(\alpha)-diol</td>
<td>21</td>
<td>1/60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$\Delta^5$-androstene-3(\beta), 7(\beta), 17(\beta)-triol</td>
<td>464</td>
<td>1/1326</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The EC$_{50}$ was calculated as the 50% activation point. The androgenicity is the testosterone EC$_{50}$ divided by the androstene hormone EC$_{50}$. This data does not normalize the cellular uptake of androstene hormones.

*Androstene Hormone Activation of the human ER$\beta$ and ER$\alpha$ receptors*

The data demonstrate that both the orientation of the hydroxyl at position C17 and the addition of the hydroxyl at position C7 affected the ability of DHEA, 17\(\beta\)-AED, 17\(\beta\)-AET and 17\(\alpha\)-AED to activate the human ER$\beta$ construct (Figure 3). The ER receptor construct contains a luciferase reporter gene that is functionally linked to an ER responsive promoter. The luciferase reading is utilized as a surrogate measure for ER binding. The EC$_{50}$ was calculated for 17\(\beta\)-estradiol as the 50% activation point. EC$_{50}$ values for the androstene hormones and 17\(\beta\)-estradiol were calculated (Table 2) and compared utilizing the ratio of the androstene hormone EC$_{50}$ to 17\(\beta\)-estradiol EC$_{50}$ (Relative Estrogenicity). All of the tested androstene hormones demonstrated a significantly (p<0.001) decreased estrogenicity compared to 17\(\beta\)-estradiol (Table 2.). 17\(\beta\)-AED had only 1/282 the ability of 17\(\beta\)-estradiol to activate the ER$\beta$. The orientation change of the hydroxyl group at position C17 of 17\(\beta\)-AED to the(\(\alpha\)) position resulted in 17\(\alpha\)-AED possessing 1/7609th the ability to activate the ER$\beta$ receptor as 17\(\beta\)-estradiol. This represents a drastic decrease in estrogenicity from the 17\(\beta\)-AED epimer. The presence of the hydroxyl group at C7 of 17\(\beta\)-AET resulted in 1/587th the ability to activate the ER$\beta$. DHEA, with a ketone group in the C17 position, possessed 1/3543rd the ability to activate the ER$\beta$. 

33
The rank order of androstene hormone activation on the human ERβ receptor can be displayed as follows: 17β-AED > 17β-AET > DHEA > 17α-AED (Figure 3).

Figure 3. Androstene Hormone Activation of the Human Estrogen Receptor Beta

Reporter cells were treated with 17α-AED or 17β-AED or 17β-AET or DHEA (n=3) at each concentration, incubated for 24 hours and then assayed for luciferase activity (n=3), incubated. Androstene hormone activity was normalized to cellular uptake. Error Bars, ± 1SD. Statistical significance, p<0.001(*) versus androstene hormone metabolites in the same concentration group, p< 0.001(¤) versus lower reacting androstene hormones in the same concentration group
These androstene hormones also specifically activated the human ERβ receptor and demonstrated the crucial effect of the (β) C17 hydroxyl group. 17β-AED and 17α-AED activated the ERβ receptor 2 and 3 orders of magnitude lower respectively than 17β-estradiol (Table 2). The 17β-AED demonstrated an estrogenicity of 1/1176 when assayed on the human ERα. Activation of the ERα receptor by 17β-AED did not become apparent until the concentration

![Graph showing activation of the human estrogen receptor alpha](image)

**Figure 4. 17β-AED Activation of the Human Estrogen Receptor Alpha**

Reporter cells were treated with 17β-AED (n=3), incubated for 24 hours, and then assayed for luciferase activity. 17β-AED activity was normalized to cellular uptake, Error Bars, ± 1SD.

reached 25 nM (Figure 4) which was 3 orders of magnitude lower than 17β-estradiol further demonstrating the weak estrogenicity displayed by these hormones at the level of the ER receptors. Finally, it should be noted that the androstene hormones only weakly activated the AR receptor and were even weaker activators of the human ER receptors.
Table 2: Relative Estrogenicity of Androstene Hormones

<table>
<thead>
<tr>
<th>Hormone</th>
<th>EC$_{50}$(nM)</th>
<th>Estrogenicity</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>0.046</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Δ$^5$-androstene-3β, 17β-diol</td>
<td>13</td>
<td>1/282</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ$^5$-androstene-3β, 7β, 17β-triol</td>
<td>27</td>
<td>1/587</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DHEA</td>
<td>163</td>
<td>1/3543</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ$^5$-androstene-3β, 17α-diol</td>
<td>350</td>
<td>1/7609</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The EC$_{50}$ was calculated as the 50% activation point. The estrogenicity is the 17β-estradiol EC$_{50}$ divided by the androstene hormone EC$_{50}$. This data does not normalize the cellular uptake of androstene hormones.

**Androstene Hormones Activation of the Human Glucocorticoid (GR) Receptor**

17β-AED, and especially 17β-AET are known to produce significantly affect glucocorticoid activity in vivo [92][78][91]. Therefore, the human GR construct response to 17β-AED, 17β-AET, DHEA and 17α-AED alone and in combination with dexamethasone was evaluated. The results showed dexamethasone alone activated the human GR receptor while 17β-AED, 17β-AET, DHEA, and 17α-AED alone were negative at all concentrations tested (Figure 5).
Figure 5. Dexamethasone or Androstene Hormone Activation of the Human Glucocorticoid Receptor

Reporter cells were treated with dexamethasone or androstene hormones alone (n=3), incubated for 24 hours, and then assayed for luciferase activity. Androstene Hormones are: DHEA, 17β-AED, 17α-AED and 17β- AET. Error Bars, ± 1SD.
The human GR receptor was then tested for activity with androstene hormones in the presence of dexamethasone. Unexpectedly, at supra-pharmacological levels of dexamethasone there was a considerable activation of the human GR receptor by 1 µM of each of the androstene hormones, which was greater than with dexamethasone alone with a higher activity when dexamethasone concentration was increased from 333pM to 1000 pM (Figure 6).

The rank order of activation of the dexamethasone-bound human GR receptor in the presence of the androstene hormones is: 17β-AET>17β-AED>17α-AED>DHEA. These data demonstrate that the C7 hydroxyl present in 17β-AET produced the strongest activation of the dexamethasone-bound human GR. The (β) C17 hydroxyl of 17β-AED produced a stronger activation than did the (α) C17 hydroxyl of 17α-AED. Thus, while 17β-AED and 17α-AED produced unique activation of the dexamethasone-bound human GR, the effect of the C17 hydroxyl group conformation was less apparent. DHEA, which has the keto group in the C17 position, possessed the least ability to activate the dexamethasone-bound human GR.
Figure 6. Androstene Hormones and Dexamethasone Activation of the Human GR Receptor

Reporter cells were treated with dexamethasone alone or a combination of dexamethasone and androstene hormone, incubated for 24 hours, and then assayed for luciferase activity. Error Bars, ± 1SD. Statistical significance, p is at least <0.05 from other androstene hormone metabolites within the dexamethasone concentration treatment group (●), p is <0.05 versus control (*), p is <0.05 from 333pM dexamethasone treatment group (●).
We next tested these effects with the dexamethasone inhibitor, cyproterone acetate to see if we could reduce or eliminate the dexamethasone and androstene hormone activation of the human GR receptor. Cyproterone acetate was selected as the inhibitor because of its unique glucocorticoid receptor inhibiting properties [178]. Cyproterone effectively inhibited the activation of the human GR receptor by dexamethasone (Figure 7). Cyproterone acetate at a concentration of 10uM was tested in the presence of the androstene hormones alone (1.0 uM) and there was no activation detected (data not shown). Androstene hormones, however in the presence of cyproterone and dexamethasone exhibited different levels of activation that were significantly increased above the dexamethasone/cyproterone alone controls (Figure 7). These results are of particular clinical significance because it demonstrates that high dose dexamethasone alters the human GR receptor to interact with other biologically active hormones at the receptor level. Importantly, dexamethasone, is known to cause adverse effects in humans [179].
Figure 7. Androstene Hormone and Dexamethasone Activation of the Human GR Receptor in the presence of Cyproterone Acetate

Reporter cells were treated with dexamethasone and cyproterone acetate alone or androstene hormones with dexamethasone and cyproterone acetate (n=3), incubated for 24 hours, and then assayed for luciferase activity. Androstene hormone activity was normalized to cellular uptake. Error Bars ± 1SD, Statistical significance, p is <0.05 from other androstene hormone metabolites within the dexamethasone treatment group (♀), p<0.05 from controls (*).
The rank order of activation of the dexamethasone/cyproterone acetate-bound human GR in the presence of these androstene hormones is: $17\beta$-AET>$17\beta$-AED>$17\alpha$-AED>DHEA. This rank order of activation on the inhibited human GR was the same as that of the uninhibited human GR indicating that the interaction of the androstene hormones and the dexamethasone/cyproterone-bound human GR was not disrupted. Because cyproterone acetate is a passive inhibitor of the human GR receptor and opposes dexamethasone through an overlapping steroid scaffold mechanism, this suggests that the androstene hormone activation is mediated by an interaction that occurs outside the dexamethasone/cyproterone acetate-bound complex [178]. Additionally, the presence of dexamethasone-bound human GR is required to observe activation by the androstene hormones while cyproterone acetate alone does not mediate this effect. Together these data suggest an indirect activation of the ligand-bound human GR receptor by $17\beta$-AET, $17\beta$-AED, $17\alpha$-AED and DHEA.

Summary

These results indicate that the position of the hydroxyl group at C17 and/or the addition of the hydroxyl group at position C7 significantly affected the ability of $17\beta$-AET, $17\beta$-AED, $17\alpha$-AED and DHEA to interact with the human estrogen, androgen and ligand-bound GR receptors. $17\beta$-AET, $17\beta$-AED, $17\alpha$-AED and DHEA were shown to interact either directly or indirectly with the human (AR, ER) and GR respectively. Importantly, $17\beta$-AET, $17\beta$-AED, $17\alpha$-AED and DHEA were shown to possess weak androgenicity and even weaker estrogenicity at the receptor level. Clinically, this is beneficial because the biological effects can be realized without unwanted androgenic or estrogenic effects.
In stark contrast to the minimal receptor activation of AR, ER and GR, these same androstene hormones produce striking biological effects in vitro and in vivo which have been attributed to activity with the AR, ER or GR receptors. Clearly, these effects may not be mediated by the direct androstene hormone interaction with the human AR, ERα, ERβ and GR receptors. Indeed, the biological mechanism, may not require AR or ER receptors to achieve significant effects [155][180]. Furthermore, the interaction with dexamethasone is indirect, occurs at high doses and is not abolished by cyproterone acetate. Taken together, the data shows that interactions of 17β-AET, 17β-AED, 17α-AED and DHEA with the human AR, ERα, ERβ and GR receptors are directed by the structure-activity of these androstene hormones with minimal androgenic, estrogenic or glucocorticoid effects and accentuates the need to further uncover the implied yet unidentified main mechanism (s) of action of these important adrenal hormones.
CHAPTER 3: Androstene Hormone Epimers Regulate ER Stress and Core Regulatory Genes in Human T98G Glioma Cells.

Introduction

High grade gliomas represent approximately 50% of the primary central nervous system (CNS) tumors with 15,000 cases diagnosed in the United States each year [181]. Grade IV glioblastomas (Glioblastoma Multiforme) are World Health Organization (WHO) classified as astrocytic neoplasms derived from the glial lineage that have progressed from Grade III (high proliferation) to Grade IV (necrotic tissue and/or angiogenic activity) [182][183][184][185]. The mean life expectancy of patients with Glioblastoma Multiforme is only 1 year from the time of initial diagnosis and only several months after progression [186]. The traditional therapy for these aggressive tumors is surgical resection followed by external beam radiation and/or chemotherapy, however, these treatments are considered palliative with only a very low survival rate. Temozolomide, an oral alkylating agent that enters the CNS, is the most commonly used chemotherapy treatment alone or in combination with compounds known to induce cell death [187][188]. Temozolomide, an oral alkylating agent, however, only improves the lifespan of malignant glioma patients by 2-3 months [187]. A major obstacle to complete tumor resection is the high invasiveness of the tumors [8].

Human derived T98G cells are a well characterized in vitro model of glioblastoma [189]. These cells were obtained from a 61 year old male and became spontaneously polyploid through culture passage [190]. T98G glioblastoma cells have been utilized as a model system for
targeting Glioblastoma Multiforme pathways [191]. T98G cells contain functional PDGF(Platelet Derived Growth Factor) receptors that can induce a malignant phenotype with sufficient stimulation [192][193]. T98G cells contain multiple mutations that have contributed to their tumorigenic properties. These mutations include a homozygous mutation for p53, CDKN2A deletion and PTEN mutation [193][194]. Transformation of T98G cells by PDGF combined with the loss of tumor suppressor activity is associated with three signaling pathways that are crucial in Glioblastoma [193].

\[ \Delta^5 \text{-androstene-3\(\beta\), 17\(\alpha\)-diol (17\(\alpha\)-AED)} \] has been shown to produce definitive type II programmed cell death in T98G cells (Figure 8) [157]. The mechanism whereby 17\(\alpha\)-AED induces oncophagy in T98G cells has previously been identified and described [195][196]. \[ \Delta^5 \text{-androstene-3\(\beta\), 17\(\beta\)-diol (17\(\beta\)-AED)} \] is the epimer of 17\(\alpha\)-AED. These naturally produced adrenal hormones are chemically identical and differ only in the stereoisomeric position of the hydroxyl group located at carbon 17 of the steroid ring structure. 17\(\beta\)-AED, in opposition to 17\(\alpha\)-AED, does not produce cell death in T98G Glioblastoma cells (Figure 8) [196]. 17\(\beta\)-AED, in fact, promotes significant biological effects including the enhancement of the immune system [81][197] even in the presence of glucocorticoids [112][109] and provides protection against lethal radiation [198]. The relationship of chemical structure to biological
Figure 8: Contrasting Effects on Cell Morphology of T98Glioma cells treated with $17\alpha$-AED or $17\beta$-AED. Prior confirmation of autophagy in cells treated with 9.5 uM of $17\alpha$-AED was performed with electron microscopy [16]. Cells were stained with hematoxylin and eosin and visualized with light microscopy at 100x. Left panel demonstrates autophagy induced by $17\alpha$-AED while the right panel demonstrates the lack of apoptosis or autophagy in $17\beta$-AED treated cells.

function is shown in Figure 9 and Table 3 lists the opposing biological functions of $17\beta$-AED and $17\alpha$-AED.
Figure 9: Structure-function relationship of the 17 hydroxyl position of the chemically identical androstenediol epimers, 17α-AED and 17β-AED results in opposing biological functions. The 17α-AED results in oncophagy (target cell specific cell death by apoptosis or autophagy) while the 17β-AED result in Immune Upregulation (adapted from Loria and Graf (2012)).

Many large-scale investigations into complex human disease have been performed and genome wide analysis of RNA expression is a common way to investigate complex human disease. It has been stated that the major challenge of these investigations is to gain relevant biological insight into these diseases [199]. 17α-AED and 17β-AED are chemically identical, produce either cell survival or irreversible death, and the biological actions of 17α-AED have
been partially identified. Clearly these epimers are ideal for monitoring the “mirror” effects observed in human Glioblastoma cells. Thus, we utilized these hormones, signal targeting microarray genes and Ingenuity IPA® networking software to demonstrate that these stereoisomers regulate core transcriptional regulators and influence critical signal transduction pathways that are critical for the death or survival of Glioblastoma.
### TABLE 3: Biological Function of 17α-AED and 17β-AED

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Model Type</th>
<th>Cell Type(s)</th>
<th>Biological Function(s)</th>
<th>References</th>
</tr>
</thead>
</table>
| 17α-AED | In vitro   | ZR-75-1 MCF-7 | 1) Inhibition of DNA Synthesis  
2) Androgen and Estrogen independent inhibition of tumor cell proliferation  
3) Combined treatment of 17α-AED and 17β-AED potentiated the effect of 17α-AED | (Huynh et al., 2000) |
|         | In vitro   | T98G U937    | 1) 17α-AED induces autophagy in the glioblastoma cell line(T98G)  
2) 17α-AED induces apoptosis in the myeloid cell line(U937)  
3) Type of cell death induced by 17α-AED is determined by the target tissue | (Loria and Graf, 2012) |
|         | In vitro   | T98G U87MG U251MG LN-18 LN-229 LN-Z308 | 1) Autophagy induced specifically through PERK/CHOP/GRP78/elf2α/BECLIN/LC3 signaling and not through IRE1 or ATF6  
2) Inhibition of elf2α in T98G cells by introduction of elf2αS51A dominant negative inhibited the induction of autophagy by 17α-AED  
3) ER stress is linked to 17α-AED induced autophagy by PERK/elf2α signaling | (Loria et al., 2012)(Jia et al., 2010) |
|         | In Vitro   | MCF-7 MDA- 231 T47D TTU-1 | 1) Enhanced radiation cytotoxicity and autophagy induction in human breast cells  
2) Estrogen independent inhibition of tumor cell proliferation  
3) Autophagy induced through PERK/CHOP/elf2α signaling | (Loria et al., 2012) |
|         | In vitro   | T98G         | 1) The position of the hydroxyl located at carbon-17 of the chemically identical stereoisomers of androstenediol dictates the biological effect.  
2) The 3-hydroxyl position was found to not influence biological effects. | (Graf et al., 2009) |
| 17β-AED | In vitro | HL-60 P388D1 | 1) Does not inhibit DNA synthesis at concentrations that produced irreversible cell death with 17α-AED
2) High doses did decrease DNA synthesis | (Huynh, P, Loria, R.M, 1997) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>ZR-75-1 MCF-1</td>
<td>1) 17β-AED shown to increase the proliferation of ZR75-1 and MCF-7 cells</td>
<td>(Poulin, R., Labrie, F., 1986) (Hackenberg, R. et al., 1993)</td>
<td></td>
</tr>
<tr>
<td>In vivo</td>
<td>CD-1 Mice</td>
<td>1) Protection from lethal infections of Pseudomonas aeruginosa and Enterococcus faecalis</td>
<td>(Ben-Nathan et al, 1999)</td>
<td></td>
</tr>
<tr>
<td>In vivo</td>
<td>Male Sprague Dawley Rats</td>
<td>1) KLF6, BCL2, p53 upregulated by 17β-AED leading to downregulation of iNOS and</td>
<td>(Kiang et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>In vivo</td>
<td>CD-1 Mice</td>
<td>1) In wound healing: countered the suppressive effect of restraint on MCP-1 and IL-1 expression in mice</td>
<td>(Head et al., 2006)</td>
<td></td>
</tr>
<tr>
<td>Ex-vivo</td>
<td>Murine Lymphocytes</td>
<td>1) Minimally counteracts hydrocortisone suppression of IL-2/IL-3 production and cell proliferation and does not cause splenocyte proliferation</td>
<td>(Padgett and Loria, 1994)</td>
<td></td>
</tr>
<tr>
<td>17α-AED/17β-AED</td>
<td>In vitro</td>
<td>Assay Cells</td>
<td>1) 17α-AED and 17β-AED differentially and directly activate human estrogen alpha, estrogen beta, androgen and indirectly activate glucocorticoid constructs at the level of the receptor in a whole cell construct with markedly decreased androgenicity and estrogenicity</td>
<td>[200]</td>
</tr>
</tbody>
</table>
Methodology

Cell culture

T98G Glioblastoma cells were culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and nonessential amino acids as adherent monolayers at 37°C, passed biweekly with trypsin in the absence of antibiotics. All tissue culture reagents and supplements were obtained from Invitrogen (Carlsbad, CA). The human T98G line was acquired from the American Type Culture Collection (Manassas, VA) and maintained by the Neuro-Oncology Research Group at the Virginia Commonwealth Medical Center, Richmond, VA.

Androstene Hormone Controls

17α-AED and 17β-AED are chemically identical and the control was running both hormones in parallel. The only difference between these hormones is the stereoisometric position of the hydroxyl located at the C17 position (Figure 9). Therefore, qPCR confirmation was not necessary as only those genes displaying opposite regulation were considered in the analysis.

The Human Signal Transduction Pathway Finder Microarray

OligoGEArray, catalogue number OHS-014, (SuperArray Bioscience Corporation, Frederick, MD) was utilized to identify genes regulated by 17α-AED and 17β-AED in T98G cells. After an overnight incubation, cell medium was replaced and the T98G Glioma cells (1x10^4/well) were cultured in a 6-well, tissue culture plate in the presence of 9.5 uM 17α-AED or 17β-AED (provided by Dr. Loria). This concentration is the IC_{50} of 17α-AED treated T98G cells. Cells demonstrated morphology consistent with prior studies. Control wells were treated
with vehicle (50% PEG 400/50% ethanol). After a 20 hour incubation, the medium was removed and RNA was extracted from cells utilizing an RNAeasy kit (Qiagen, Valencia, CA) and quantitated spectrophotometrically. 1 ug/ml RNA was utilized with 3 uL GEAprimer Mix and 6 ul H2O in the annealing mixture for probe synthesis. This mixture was heated at 70°C for 3 min then cooled to 42°C and then incubated at 42°C for 2 min. Labeling mix was then prepared by mixing 4ul of 5X GEA labeling Buffer, 3ul of [a-32P] dCTP (10mCi/ml), 1ul RNase inhibitor, 1ul Reverse transcriptase (50U/ml) and 1ul of RNase free H2O. The labeling mix was then added to the RNA and the labeling reaction was run for 25 min at 42°C. The reaction was stopped with 2 ul of stop solution then denatured with 2ul of denaturing solution at 68°C for 20 min after which probe neutralization was performed with 20 ul of neutralization solution at 68°C for 10 min. Labeled probes were mixed with hybridization buffer and added to a prehybridized membrane and incubated overnight at 60°C. The hybridized membrane was washed 2x with SSC, 1% SDS for 10min at 60°C then washed once with 0.1x SSC, 0.5% SDS for 10 min at 60°C (Figure 10).
Figure 10: Human signal Transduction Pathway Finder Microarray

Human T98G Glioma cells were treated with vehicle or neuro-steroid for 20h. Total RNA was used to generate cRNA which was them used to probe the microarrays which contain DNA oligos from genes related to cell stress, cell toxicity, drug resistance and drug metabolism. Spots that are contained in the heavy circles represent housekeeping genes (\(\beta\)-actin, \(\beta\)2-microglobulin, ribosomal protein 27a, etc.) used to normalize the data. The light circle represents an internal control for orientation.
IPA Network Generation and Analysis

Network Generation

An Ingenuity IPA® core analysis was performed where the identified target genes overlaid onto a global molecular network developed from information contained in the IPA database. The Ingenuity Knowledge Base Includes: Data Sources scanned included Ingenuity expert findings®, mi records, TaRbase, TargetScan Human, BIND, BIOGRID, Cognia, DIP, INTACT, Interactome studies, MINT, MIPS, ClinicalTrials.gov, GeneOntology, GVK Biosciences, Kyoto Encyclopedia of Genes, miRBase and the Obesity Gene Map.

Network Score Statistics

The network score is a statistical numerical value used to estimate how well the network eligible genes match the Ingenuity Knowledge Base genes. The score takes into account the total number of network eligible molecules, network size, and the total number of possible associated molecules in the Ingenuity Knowledge Base. The Network Score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's Exact Test. For example, a network of 35 molecules has a Fisher Exact Test result of $1 \times 10^{-8}$. The network’s Score = -log (Fisher's Exact test result) = 8. This is interpreted as there is a 1 in a 100 million chance of obtaining the 35 molecule network containing network eligible molecules randomly that could be in networks generated from the Ingenuity Knowledge Base.
Results and Discussion

The Human Signal Transduction Pathway Finder Microarray

Microarray results were imaged on a phosphoimager. The acquired images were extracted and converted into raw signals using GEArray ScanAlyze software. The procedure is found at: (http://www.sabiosciences.com/manuals/GEArrayAnalyzerTutorial.ppt#278, 27, Chart). Probe sets were compared and normalized to β-actin controls (Figure 11).
Figure 11: Contrasting Effects on Gene Expression in T98Glioma cells treated with 17α-AED or 17β-AED. A) Decreased gene expression in T98G cells treated with 17α-AED. Green marks below the vertical represent genes in T98G cells treated with 17α-AED that have a greater than 5-fold decrease in expression as compared to T98G cells treated with vehicle. Data normalized to β-actin gene expression. B) Same as in A, however T98G cells were treated with 9.5 uM of 17β-AED. Numerous genes show a more than 5-fold increase in expression as indicated by red marks above the vertical.
Network Analysis

All 26 target gene probesets were mapped to their corresponding gene in the IPA Knowledge Base through the GenBank ID number. The IPA core analysis yielded a total of five, 35 molecule networks which were based on a score. The score takes into account the total number of network eligible genes, network size, and the total number of possible associated molecules in the Ingenuity Knowledge Base. The network scores for the network analysis are as follows: network 1: (score: 16), network 2: (score: 12), network 3: (score: 12), network 4: (score: 10), network 5: (score: 1). It should be noted that network 5 (1 in 10 chance of occurring randomly) is not statistically significant while the first 4 networks are extremely statistically significant. Network 1 has a 1 in 10 zillion chance of being random, networks 2 and 3 have a 1 in 1 trillion chance of being random and network 4 has a 1 in 10 billion chance of being random.

Target Genes

The term "Target Gene(s)" refers to the total geneset whose expression 1) was regulated down (negative), at a minimum of 5 fold by 17α-AED and/or regulated up (positive), at a minimum of 5 fold by 17β-AED and 2) resulted in a total difference in gene expression that is, at a minimum, an order of magnitude) between 17α-AED downregulated and 17β-AED upregulated expression of that gene. Because 17α-AED and 17β-AED are chemically identical this 10 fold or greater significance indicates that this expression may be related to the opposing biological functions of survival or irreversible cell death. We have identified 26 target genes in human T98G glioma cells (Table 4).
### TABLE 4: 17α-AED/17β-AED Target Genes in T98G glioma cells

<table>
<thead>
<tr>
<th>GENE</th>
<th>GENE BANK ID</th>
<th>DESCRIPTION</th>
<th>17α-AED</th>
<th>17β-AED</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP2</td>
<td>NM_001200</td>
<td>bone morphogenetic protein 2</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>BRCA1</td>
<td>NM_007294</td>
<td>breast cancer 1, early onset</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>CCL2</td>
<td>NM_002982</td>
<td>chemokine(C-C motif) Ligands 2</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>CTSD</td>
<td>NM_001909</td>
<td>cathepsin D</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>NM_000389</td>
<td>cyclin-dependent kinaseinhibitor 1A(p21, Cip1)</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>CDKN2D</td>
<td>NM_001800</td>
<td>cyclin-dependent Kinase inhibitor 2D(p19, inhibits CDK4)</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>CDK2</td>
<td>NM_001798</td>
<td>cyclin-dependent kinase 2</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>CSN2</td>
<td>NM_001891</td>
<td>casein beta</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>EGFR</td>
<td>NM_005228</td>
<td>epidermal growth factor receptor</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>EGR1</td>
<td>NM_001964</td>
<td>early growth response 1</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>GADD45A</td>
<td>NM_001924</td>
<td>growth arrest and DNA-damage-inducible, alpha</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>GYS1</td>
<td>NM_002103</td>
<td>glycogen synthase 1(muscle)</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>HOXB1</td>
<td>NM_002144</td>
<td>homeobox B1</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>ICAM1</td>
<td>NM_000201</td>
<td>intercellular adhesion molecule 1</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>IGFBP4</td>
<td>NM_001552</td>
<td>insulin-like growth factor binding protein 4</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>IRF1</td>
<td>NM_002198</td>
<td>interferon regulatory factor 1</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>JUN</td>
<td>NM_002228</td>
<td>jun proto-oncogene</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>NAB2</td>
<td>NM_005967</td>
<td>NGFI-A binding protein2 (EGR1 binding-protein 2)</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>NM_020529</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>ODC1</td>
<td>NM_002539</td>
<td>ornithine decarboxylase 1</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>PMEPA1</td>
<td>NM_020182</td>
<td>prostate transmembrane protein, androgen-induced 1</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>TP53</td>
<td>NM_000546</td>
<td>tumor protein p53</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>TP53I3</td>
<td>NM_004881</td>
<td>tumor protein p53 inducible protein 3</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>WNT1</td>
<td>NM_005430</td>
<td>wingless-type MMTV</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
</tbody>
</table>
General Discussion

Tumor cells are embedded in a hostile environment that is constantly challenged by chronic metabolic stress conditions that favor the activation of adaptive mechanisms in response to stress which includes autophagy [201][202]. The surrounding environment can influence the type and amount of protein that need to be folded in the ER. Unfolded protein responses (UPR) are initiated when misfolded proteins are sensed in the endoplasmic reticulum (ER). Previously it was shown that the same agent, the neurosteroid, 17α-AED can induce irreversible type I cell death (apoptosis) in myeloid cells and type II cell death in human glioma cells (autophagy) [154].

It was shown that 17α-AED-induced autophagy in T98G cells occurred through a specific partial unfolded protein response (UPR) [161] where the mechanism appeared to evolve from PERK activation and proceed to the phosphorylation of the eukaryotic translation initiation factor 2α. Specifically, eIF2α phosphorylation was shown to induce CHOP (CCAAT/enhancer
binding protein homologous protein (CHOP) and the ER chaperone proteins such as glucose-regulated protein of 78kDa (GRP78)) [161]. The response was also shown to be independent of the double stranded RNA-activated protein kinase (PKR) and the general control non-derepressable 2 (GCN2) protein kinase [161]. These molecular events ended in the induction of beclin-1 and microtubule light chain 3 (LC3) cleavage resulting in irreversible autophagy. Thus, in TG98 Glioblastoma 17α-AED induces an unfolded protein response through PERK activation with resultant autophagy (Figure 12). 17β-AED, the chemically identical epimer, appears to produce a different response that results in survival.

Autophagy ensues when beclin-1 forms a complex with Vps34 (PI3KClass III) [203]. BCL-2 prevents autophagy by forming a mutually exclusive complex with beclin-1 through the BH3 domain and inhibiting beclin-1 [204]. BCL-2 is an anti-apoptotic member of the BCL-2
family which binds to the outer membrane of the mitochondrion and serves to sequester BH3-only proteins through direct binding. BCL-2 acts as a shield against these BH-3 only pro-apoptotic agents which attack and destabilize the mitochondrion [205]. When BCL-2 cannot effectively bind beclin-1 then beclin-1 forms a complex with Vps34 (PI3KClass III) which activates membrane nucleation with ensuing autophagy [203].

Network analysis revealed that breast cancer early onset (BRCA-1) and BCL2 were main hubs that were directly associated in Network 1 (most significant) which demonstrate their main role in T98G stress response directed by 17α-AED and 17β-AED. Network analysis also demonstrated that GADD45 and IRF-1 were clustered together in network 4 while CDKN1A was clustered in network 2. These observations indicate that while CDKN1A, GADD45 and IRF-1 are BRCA-1 targets they are separated functionally from BRCA-1 by association with different genes.

17α-AED induces CHOP which is known to downregulate BCL-2 expression [161][206]. 17α-AED downregulated both BCL-2 and BRCA-1 suggesting that the biological response initiated by 17α-AED extends beyond downregulation of BCL-2. Conversely, 17β-AED upregulated both BCL-2 and BRCA-1. Overexpression of BRCA-1 was shown to produce an apoptotic phenotype and correlated with GADD45 production [207]. 17β-AED upregulated BRCA-1, CDKN1A, GADD45 and IRF-1 and did not produce cell death. These are target genes that are associated with DNA repair, cell cycle regulation and immunity [208]. Upregulation of these genes have been shown to be BRCA-1 dependent [209][210]. CDKN1A expression, however, is not always obtained in the presence of BRCA-1 [211].

Recently, it was shown that BCL-2 co-localizes with BRCA-1 to the mitochondrial and endoplasmic reticulum endomembranes [41]. BCL-2, while protective, will oppose genome
stability by binding to and preventing nuclear localization of BRCA-1 which results in an apoptotic phenotype [41]. BCL-2 will preferentially bind to Beclin-1 and inhibit autophagy [212]. Subsequently, the GADD45, CDKN1A and IRF-1 upregulation by 17β-AED indicates nuclear BRCA-1 activity despite upregulated BCL-2. 17α-AED directs a downregulation of BRCA-1 and BCL-2, thus targeting the beclin-1/Vps34 interface and irreversible autophagy (Figure 13). BRCA-1 is considered a tumor suppressor. Downregulation of BRCA-1 with activation of irreversible cell death demonstrates that while BRCA-1 is involved in these processes it is not required to achieve cell death.
Figure 13. Stress Induced Responses Directed by 17α-AED and 17β-AED

A) Specific 17α-AED-directed, PERK activated stress response leading to autophagy B) BRCA-1 and BCL-2 are induced by 17β-AED leading to autophagy inhibition, DNA repair, growth control, immune response and survival. Double slash // indicates a complex.
The regulated expression of CDKN1A by BRCA-1 has been shown to occur in p53 dependent and independent manner. p53-independent induction of CDKN1A may occur through an interaction that occurs through a binding interaction of BRCA-1 with C-terminal binding protein (CtBP) and retinoblastoma binding protein 8 (CtIP) [213]. This interaction normally inhibits the activation of BRCA-1 but in the presence of stress BRCA-1 is released from this inhibitory interaction to activate gene transcription [213]. In another study, it was shown that BRCA-1 augments the p53 transcription factor but selectively induces genes involved in DNA repair and arrest of the cell cycle but not genes that direct apoptosis for target gene induction [214]. The absence of apoptosis from 17β-AED induced CDKN1A suggests that the main function of 17β-AED in T98G cells depends on BRCA-1 in a p53-dependent way (Figure 14).

Further support is demonstrated by the 17β-AED directed upregulation of BRCA-1, p53, GADD45 and TP53I3 (PIG3) which indicates the stabilization of p53 with expression of DNA repair genes. Importantly, this is occurring in the presence the p53 dual mutation that is present in T98G cells [193][194]. Interestingly, these observations may indicate a role for 17β-AED in BRCA-1 mediated tumor suppression.
Figure 14. BRCA-1 directed, p53 dependent transcriptional response induced by 17β-AED.

A) BRCA-1 directed, p53 independent transcriptional response induced by stress. B) BRCA-1 directed, p53 dependent transcriptional response induced by 17β-AED.

We show that 17β-AED and 17α-AED regulated p53 in an opposing manner. Network analysis of 17β-AED and 17α-AED target genes demonstrated that AKT was the central signaling hub of the top ranked network indicating its importance and significance. The Wnt
pathway was previously shown to act in conjunction with AKT to stimulate phosphorylation and inactivation of GSK3B [215]. 17α-AED downregulated WNT1 thus supporting activation of

Figure 15. Opposing Regulation of p53 Expression Induced by 17α-AED or 17β-AED through WNT1, AKT and GSK3β.
GSK3B. Additionally, activation of PERK by 17α-AED also supports the activation of GSK3B kinase further reinforcing the reduction of p53 [216]. Conversely, 17β-AED upregulated p53 and WNT1 and the latter is known to decrease the activity of GSK3B through canonical WNT signaling [217]. Thus, this evidence suggests that 17β-AED opposes the action of 17α-AED by influencing the expression of WNT1 and subsequently influencing the AKT/GSK3B axis resulting in the opposing transcription of p53 (Figure 15).

Network analysis demonstrated that the target molecules of AKT in the dataset (BCL2, BMP2, CCL2, CDK2, CDKN1A, CSN2, EGR1, and TP53) were inconsistent with predicted findings for gene expression induced by 17β-AED and 17α-AED. Furthermore, the predictions of AKT target molecules were also opposing demonstrating that AKT is modulated by both steroids. It is important to mention that the PTEN tumor suppressor is mutated in T98G cells [193]. This mutation is important twofold. First, PTEN is a tumor suppressor that induces autophagy [218] and second, PTEN can do this by activating the PERK/elF2α independently of its phosphatase activity [219]. This demonstrates that 17α-AED induces irreversible autophagy through PERK and elF2α independently of PTEN. It is unclear at this point if the PTEN mutation in T98G cells affects the BRCA-1 directed, p53 dependent response induced by 17β-AED.

An important observation from the microarray is that 17β-AED induced upregulation of both CDKN2D and p53. A p53 core regulator complex composed of MDM2, p53, CDKN2A and E2F was shown to regulate genes controlling G1-S and G2-M cell cycle arrest, apoptosis, inhibition of angiogenesis/metastasis and DNA repair (Figure 16A) [220]. CDKN2A and/or CDKN2D will bind to and inactivate MDM2, a ubiquitin ligase that will ubiquitinate p53 resulting in increased levels and stabilization of the p53 protein.
Figure 16: Core Regulatory Unit Governing Cell Cycle Arrest, Apoptosis, Inhibition of Angiogenesis and Metastasis and DNA Repair

A) Normal Core Regulatory Unit  
B) T98G core regulatory unit disrupted by mutations in p53 and CDKN2A  
C) 17β-AED induced CDKN2D partially restructures the core regulatory unit  
D) 17α-AED downregulates p53 and CDKN2D disrupting the core regulatory unit leading to the induction of irreversible autophagy.
CDKN2A and/or CDKN2D complexes are positively regulated by E2F1[223]. CDKN2A is detected in T98G cells which affects the CDKN2A/p53 axis as well as CDKN2A/Rb axis (Figure 16B).

DNA damage will activate p53 but not CDKN2A/CDKN2D [ 224][225]. Thus, 17β-AED and not DNA damage upregulated CDKN2D to restructure the p53 core regulatory unit (Figure 16C). CDKN2A is associated with tumor suppressor functionality through p53 dependent apoptosis whereas CDKN2D is not [226][227]. However, p53-negative cell lines are resistant to CDKN2D-induced growth arrest [225]. Furthermore, loss of CDKN2D in the presence of functional CDKN2A results in tumors early in life [225]. Together, these observations demonstrate that some functionality is restored to the p53 core regulatory unit through CDKN2D. However, at this point it is unclear how the functionality is affected because CDKN2A is deleted in T98G cells. In contrast to 17β-AED, 17α-AED downregulated p53 and CDKN2D. These observations demonstrate that 17α-AED influences the p53, CDKN2D and E2F core regulatory unit by downregulating p53 and CDKN2D while 17β-AED restructures this core regulatory unit through upregulation of p53 and CDKN2D.

Free E2F is a link between p53 and the retinoblastoma tumor suppressor (Rb) [227]. The cell cycle normally progresses when Rb is inactivated by phosphorylation that is catalyzed by CDK-cyclin complexes [228][229]. Rb and E2F were associated with CDK2 (a target gene) in the core network analysis. CDK2 is present within the CDK-cyclin complexes that drive the cell through G1-S phases of the cell cycle. CDK2 is a central gene that is known to be necessary to facilitate the hyperphosphorylation of the Rb pocket after a priming phosphorylation by CDK4/6 leading to the inactivation of Rb and the release of E2F and thus CDK2 is required for inactivation of the Rb protein [227].
17β-AED induces upregulation of CDK2 thus making it possible for the inactivation of Rb through CDK2-cyclin complexes. In opposition to 17β-AED, 17α-AED downregulated CDK2 and thus negatively influenced the primary phosphorylating complexes related to CDK2 that would both drive the cell through G1-S and inactivate Rb by phosphorylation. It has been demonstrated that Rb-E2F is a rheostat that is modulated by phosphorylation thus playing an integral part in the induction of autophagy [230][231]. Here, we show that 17α-AED induced significant downregulation of CDK2, p53 and BCL2 transcription which is consistent with inhibition of the cell cycle, activation of the Rb complex and the strong induction of autophagy which was clearly demonstrated to occur (Figure 16D). Furthermore, it has been demonstrated that Rb/E2F plays a direct role in the regulation of Hox genes [232]. The fact that HoxB1 was identified as a target gene provides further support that RB/E2F is a target of 17β-AED and 17α-AED.

Finally, free E2F produced through Rb phosphorylation has been shown to produce p53 dependent apoptosis mediated through CDKN2A and p53 independent apoptosis mediated through TRAF2 [227]. This observation has two important implications. First, BIRC2 and BCL2 are both 17β-AED target genes and thus, indicate the presence of anti-apoptotic genes that prevent death derived from the intrinsic and extrinsic pathways in T98G cells. Second, p53 dependent apoptosis is not produced in T98G cells by either 17β-AED or 17α-AED. Therefore, 17β-AED and 17α-AED influence the Rb-E2F rheostat; however, other additional cellular transcriptional controls coordinate and integrate the biological outcome. More studies will need to be performed to further elucidate the action and effects of the specific cell-directed stress responses induced by the presence of 17α-AED and 17β-AED.
Summary

Targeting cancer through main pathways is fraught with difficulties [233]. 17β-AED and 17α-AED are chemically identical stereochemical epimers that differ only in the position of the hydroxyl at carbon 17 of the cycloperhydrophenanthrene ring structure (Figure 1). This report demonstrates, for the first time, that naturally occurring, chemically identical adrenal hormone (17β-AED or 17α-AED) treatments resulted in the induction of significant, specific yet differential transcriptional responses to stress that, despite the presence of major mutations, positively affected the core transcriptional regulatory unit in T98G cells as well as the three critical pathways inherent to Glioblastoma cells (Figure 17). 17α-AED treatment resulted in type II programmed cell death while 17β-AED treatment resulted in DNA repair and growth control.

Schematic diagram of the primary sequence alteration for components of the CDKN2A/MDM2/p53 axis, Ras/PI3K axis, and CDKN2A/CDK4/RB signaling pathways in T98G cells are shown. Light gray with black lettering indicates inhibiting genetic alterations in the T98G cells. Dark gray with white lettering indicates a functional mutation differing from the wild-type. Block Crosses indicate the affected biofunction. 17β-AED affects the CDKN2A/MDM2/p53 axis by up regulating CDKN2D and p53. 17α-AED affects this axis by downregulating the mutated p53 and CDKN2D thus the activity of 17α-AED does not require the CDKN2A/MDM2/p53 axis. Both 17β-AED and 17α-AED affect the Ras/PI3K axis by modulating AKT. 17α-AED phosphorylates eIF2α and thus does not require PTEN for this function. 17β-AED upregulates CDK2 while 17α-AED downregulates CDK2 and general translation. Thus both hormones regulate the RB/E2F rheostat without the requirement of CDKN2A.
These observations demonstrate that 17α-AED affects the 3 main signaling pathways without the requirement for the mutated genes in these pathways resulting in irreversible cell death while 17β-AED induces a cell-directed transcriptional program targeting DNA repair and growth control despite the presence of the mutations in these 3 pathways.
CHAPTER 4: Pressure Ulcers in Patients with Spinal Cord Injury; Microarray and Network Analysis

Introduction

Chronic non-healing wounds, primarily pressure ulcers, are a major clinical challenge in the long-term care of patients with spinal cord injury. Clinical evidence demonstrates that chronic pressure ulcers are a major source of morbidity and mortality, having a significant impact on spinal cord injury (SCI) patients’ health and health care resource expenditures. Chronic pressure ulcers may potentially occur in any SCI (spinal cord injury) patient, as a result of denervation of skin below the lesion level and impaired mobility.

Wound healing is a multifactorial process which has been elucidated by state-of-the-art wound care research. Regulation of normal wound healing depends largely on the interactions among multiple cell types, which includes immune cells (neutrophils, monocytes, lymphocytes and dendritic cells), endothelial cells, keratinocytes and fibroblasts. Those cells undergo marked changes in gene expression and phenotype, leading to cell proliferation, differentiation and migration [234-236]. If this response is successful, these processes will be shut down in a precise sequence in the ensuing days as healing progresses [235].

In SCI patients, a common and frustrating occurrence is the frequency of skin breakdown, particularly under bony prominences. A further problem with skin breakdown in persons with SCI is the slow healing rates (sometimes in excess of 1 year) in comparison with wounds in other
clinical scenarios. Over the past 30 years, research has been performed to try to uncover metabolic and physiological differences between the skin above and below the level of the SCI [237]. In SCI related chronic pressure ulcers, the normal healing process is significantly altered and delayed, which may result from multiple physiologic deficiencies in denervated skin. Several studies have shown that molecular events may be the crucial factors resulting in collagen and extracellular matrix (ECM) depletion, decreased activity and migration of fibroblasts, and wound-healing delays [237-242]. However, the precise biologic processes involved in prolonged SCI-related wound healing are still not completely understood.

Microarray technology has been utilized to examine the expression of a large number of genes in an experimental condition simultaneously [243]. It has been problematic, however, to ascertain biological meaning from the use of microarray alone. Here we utilize whole genome microarray and IPA® network analysis to identify genes that are different and biologically significant between chronic SCI related pressure ulcers and normal skin controls. In order to further characterize the transcriptional program within the chronic pressure ulcer edges, we applied targeted network analysis of known stress related genes that are transcriptionally regulated by androstene hormones to identify core transcriptional regulators associated with chronic pressure ulcer and the normal skin controls genes. These results provide a new understanding of the molecular mechanisms that differentiate chronic pressure ulcers from normal skin during wound healing.
*Materials and Methods*

*Protection of Human Subjects*

The study was approved by the Investigative Review Board Human Subjects Subcommittee at the Dallas VA Medical Center and was conducted in accordance with its regulations. To minimize risk of disclosure to others and discrimination or stigmatization, measures were taken to ensure confidentiality. Each participant was assigned a confidential code number to be used by staff when collecting and reporting information. Research staff used coded forms and confidential code numbers for study participants when collecting and processing test data. Laboratory samples and results were identified only by confidential code numbers. At no time did the project release medical or laboratory information that could in any way be linked to a particular study participant. All data used in this transcript for publication of the findings from the study were presented in aggregates and without the identities of individual participants.

*Recruitment and consent procedures*

Six males, aged 20 - 70, with paraplegia or tetraplegia hospitalized with chronic pressure ulcers in a VA SCI center were selected for this research. All patients were receiving at least daily dressing changes according to their inpatient wound care orders. Explanation was given to patients who participated in the study and any questions or concerns they might have during the study were addressed as well. A formal written consent form was given to them to read. All subjects signed the IRB-approved consent form prior to any interventions taking place. Time was provided for review so that the client could share his intention to participate with significant others or family members prior to enrollment.
Specimens

Two or more skin specimens, about 100 mg each, were excised using a scalpel from each of six patients. One or more samples were taken directly from the edge of the wound and another from normal skin above the neurologic level of injury (suprascapular region). All samples were removed immediately upon excision. Six edge specimens were obtained in patients who had pressure ulcers which lacked viable granulation tissue, i.e. had a flat, smooth appearance. All specimens were put in 5ml of RNA later (Ambion) solution and kept in refrigerator before submission for RNA extraction. Both normal and wound tissues were de-identified prior to transporting to the lab for further analysis.

Total RNA Extraction from Skin Tissues

Total RNA was isolated from skin specimens using the Lipid Tissue mini kit (Qiagen) according to manufacturer’s instructions. Briefly, 100mg tissue was homogenized in 1ml QIAzol Lysis Reagent. After addition of 200µl chloroform and shaking by hand, the homogenate is separated into aqueous and organic phases by centrifugation. The upper, aqueous phase is transferred to another tube, and an equal volume of ethanol is added to provide appropriate binding conditions. The sample was then applied to the RNeasy spin column, where the total RNA bound to the membrane and phenol and other contaminants were efficiently washed away. High-quality RNA was then eluted in 100µl RNase-free water. The RNA concentration was measured by Spectrophotometer (Nano Drop) and RNA quality was checked by Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA).
Microarray analysis using Illumina Human-6 BeadChip arrays

a. cRNA amplification and probe labeling: cRNA was amplified and labeled with biotin for each sample using MessageAmp II-biotin enhanced kit according to manufacturer’s manual (Applied Biosystems). Briefly, 200ng total RNA was used for the double strand DNA synthesis with T7-oligo (dT) primer and followed by in vitro transcription (IVT) reaction to amplify cRNA and incorporate biotin into the synthesized cRNA probe. The cRNA probe was then purified and quantified by Spectrophotometer. Three labeling replicates were done for each sample.

b. Illumina Human-6 BeadChip hybridization: Biotinylated cRNA probe was hybridized to the Illumina Human-6 BeadChip Arrays (Illumina, Inc., San Diego, CA), 1,500ng labeled cRNA was used for hybridization for each array. The hybridization, washing and scanning were performed according to the manufacturer’s manual instruction. A total of 36 arrays were used for 12 samples (each sample has 3 replicates). Network analysis was performed on these whole genome microarrays and the microarray expression data for the 16 genes identified in the most significant network are represented in Table 5. Additional information on the genes associated with UBC and that are unique to normal skin edges in network 1 can be found in Appendix 4A. Additional information on the genes associated with UBC that are unique to chronic pressure ulcers in network 1 can be found in Appendix 4B. Information on UBC-associated genes that are shared by normal skin and chronic pressure ulcer can be found in Appendix 4C.
Table 5: Whole Genome Microarray Network Analysis: Ubiquitin C-centered Networks

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Skin Control*</th>
<th>Chronic Wound*</th>
<th>Fold Change</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CERCAM</td>
<td>9</td>
<td>117.91</td>
<td>341.06</td>
<td>2.39</td>
<td>GATCAGATTCCCTAAGGCTCAGACGAGCCAGCCAGCCAG</td>
</tr>
<tr>
<td>LMF2</td>
<td>22</td>
<td>266.45</td>
<td>329</td>
<td>1.23</td>
<td>CTCCTGGGAGGATCCACATGGCTAGCGCAGAGGAAAAG</td>
</tr>
<tr>
<td>CLIP3</td>
<td>19</td>
<td>306.07</td>
<td>346.49</td>
<td>1.13</td>
<td>CAGCTGAGACTTGGCCTGGCATGTGACGACCCCTGCCC</td>
</tr>
<tr>
<td>SLC22A17</td>
<td>14</td>
<td>251.12</td>
<td>306.03</td>
<td>1.22</td>
<td>CCACGGGCTCCTGGCCTGGCTCCTGGCTCCTGGCTCCTGG</td>
</tr>
<tr>
<td>UBAP1</td>
<td>9</td>
<td>242.48</td>
<td>371.19</td>
<td>1.53</td>
<td>CCAAGCCGCTGGCTGGCCGTTCGGT</td>
</tr>
<tr>
<td>RNF24</td>
<td>20</td>
<td>136.41</td>
<td>137.2</td>
<td>1.01</td>
<td>ATCATACCTTTTGATCAGACAGTTGAGCCCCCAAG</td>
</tr>
<tr>
<td>RNF145</td>
<td>5</td>
<td>149.01</td>
<td>270.1</td>
<td>1.82</td>
<td>GCCTGGGACATGGGGTGGCCCTGGGCTGAGACGAC</td>
</tr>
<tr>
<td>C8orf76</td>
<td>8</td>
<td>330.45</td>
<td>456.72</td>
<td>1.38</td>
<td>TCACCAGAGGGAAAGTGTGGGTGGCTGGTGAG</td>
</tr>
</tbody>
</table>

**Chronic Pressure Ulcers**: 8 Genes Unique to Ubiquitin-Centered Network 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Skin Control*</th>
<th>Chronic Wound*</th>
<th>Fold Change</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPRO</td>
<td>1</td>
<td>2098.77</td>
<td>2339.86</td>
<td>1.11</td>
<td>ACTCAGAAGATCTGCACTGCCACCCCCCAGCATGGT</td>
</tr>
<tr>
<td>NCCRP1</td>
<td>13</td>
<td>1556.09</td>
<td>3275.19</td>
<td>2.10</td>
<td>CACGCTGACGGAGCTAGCTAGGGGAGGAGCCCAC</td>
</tr>
<tr>
<td>THUMP1</td>
<td>16</td>
<td>8.98</td>
<td>1.82</td>
<td>-4.93</td>
<td>GGCAGTCGGAAGAGAAATGCCCCCATAT</td>
</tr>
<tr>
<td>DEF6</td>
<td>13</td>
<td>306.84</td>
<td>263.55</td>
<td>-1.16</td>
<td>ACAGGGGATGAAAGGAGACAGAT</td>
</tr>
<tr>
<td>MMGT1</td>
<td>X</td>
<td>327.3</td>
<td>282.55</td>
<td>-1.16</td>
<td>GATCTGGGTGGGCTCTCAGTGCTGGTGGGAC</td>
</tr>
<tr>
<td>BEX4</td>
<td>X</td>
<td>1378.96</td>
<td>1050.86</td>
<td>-1.31</td>
<td>GCAACGCTCTCTTGGTGCTGGTGGTGGT</td>
</tr>
<tr>
<td>RILPL1</td>
<td>12</td>
<td>21.77</td>
<td>18.35</td>
<td>-1.19</td>
<td>TCCACGACCGAGAACAGAGGGGTGAGTGG</td>
</tr>
<tr>
<td>SECISBP2L</td>
<td>9</td>
<td>343.96</td>
<td>246.84</td>
<td>-1.39</td>
<td>ACAGCTGAGGTCCAGAAGGGTGGTGGG</td>
</tr>
</tbody>
</table>

Normal skin and chronic pressure ulcer raw data represent the average fluorescence signal intensity measured in fluorescence units [244]. Positive fold change is an increase in signal intensity from the skin control to the chronic pressure ulcer and is calculated as the chronic pressure ulcer fluorescent units divided by the skin control fluorescent units. Negative fold change is a decrease in signal intensity from the skin control to the chronic pressure ulcer.
**Microarray data analysis**

The microarray data were extracted using BeadStudio v3.1 software provided by the manufacturer. The data were background subtracted and normalized using Cubic Spline algorithms. Statistical analysis was performed by comparing the samples between each group using the Student’s t test with the Bonferroni Correction. All samples and replicates within each category were pooled as a group.

**Network Analysis**

**Network Core Analysis**

An Ingenuity IPA® core analysis was performed where the identified target genes were overlaid onto a global molecular network developed from information contained in the IPA database. The Ingenuity Knowledge Base Includes: Data Sources scanned included Ingenuity expert findings®, mi records, TaRbase, TargetScan Human, BIND, BIOGRID, Cognia, DIP, INTACT, Interactome studies, MINT, MIPS, ClinicalTrials.gov, GeneOntology, GVK Biosciences, Kyoto Encyclopedia of Genes, miRBase and the Obesity Gene Map.

**Network Statistics (Score)**

The network score is a numerical value used to estimate how well the network eligible genes match the Ingenuity Knowledge Base genes. The score takes into account the total number of network eligible molecules, network size, and the total number of possible associated molecules in the Ingenuity Knowledge Base. The Network Score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's Exact Test. For example, a network of 35 molecules has a Fisher Exact Test result of $1 \times 10^{-8}$. The network’s Score = -log (Fisher's Exact
test result) = 8. This is interpreted as there is a 1 in a 100 million chance of obtaining the 35 molecule network containing network eligible molecules randomly that could be in networks generated from the Ingenuity Knowledge Base.

**Core regulator Analysis of Chronic Wounds**

To evaluate and identify associations with core transcriptional regulators involved in stress we analyzed each gene that was different between the UBC centered Network 1 of the Normal Skin Control and the UBC centered Network1 of the Chronic Pressure Ulcer by running individual IPA®core analyses of each gene, its known binding partners identified in Ingenuity® and known ER (endoplasmic reticulum) stress sensors (ERN1, PERK, and ATF6)[245], and known genes associated with programmed cell death (PI3KC3, beclin-1, SQSTM1)[246].

Δ⁵-androstene-3β, 17α-diol (17α-AED) has been shown to produce definitive type II programmed cell death in T98G cells [157]. The mechanism whereby 17α-AED induces oncophagy in T98G cells has previously been identified and described [195][196]. Δ⁵-androstene-3β, 17β-diol (17β-AED) is the epimer of 17α-AED. These naturally produced adrenal hormones are chemically identical and differ only in the stereoisomeric position of the hydroxyl group located at carbon 17 of the steroid ring structure. 17β-AED, in opposition to 17α-AED, does not produce cell death in T98G Glioblastoma cells [196]. 17β-AED, in fact, promotes significant biological effects including the enhancement of the immune system [81][197] even in the presence of glucocorticoids [112][109] and provides protection against lethal radiation [198].

We have prior shown that these two androstene hormones regulate 26 genes related to regulation of ER stress which include or are associated with core transcriptional regulators. 17α and 17β-AED regulate these genes in an opposing manner that is at least 1 order of magnitude different. Thus these 26 genes are intricately involved in the modulation of the stress response...
and these 26 genes (Table 3) represent a set of regulatory genes that are involved in the cell directed decision to upregulate immunity, repair genomic material or self destruct through programmed cell death mechanisms. Both 17β-AED and 17α-AED regulated a specific cell directed response to stress. Because Chronic wounds represent a stressed cellular environment it was imperative to evaluate the transcriptional regulation of the 26 genes and identify the association of these core regulators with genes that were identified as significant from the network analysis of the whole genome array within the chronic wound edges.

Results and Discussion

Chronic pressure ulcers represent a stressful environment for the cells residing there. Environmental changes cause cells to alter collections of expressed proteins in order to maintain homeostasis. Protein expression patterns are known to be modified in stressed conditions through transcriptional, translational and post-translation mechanisms [247]. Cellular functions including cell survival, death, proliferation, differentiation are realized through the transcription, translation and degradation of large multi-protein complexes. The modular components must either be sustained or degraded based on cellular control. It is now understood that the cellular timescale does not always apply to the abrupt appearance or disappearance of the rate limiting components of transcription and translation molecular "machinery". The ubiquitin proteosomal system, by regulating activating and deactivating regulators through post-translational degradation of cellular proteins, centrally orchestrates cellular functions [248].

Ubiquitin is a highly conserved protein consisting of 76 amino acid that targets substrate proteins for degradation through the 26S proteosome [11]. The ubiquitin proteosomal system has emerged as the principal system of protein fate inside cells [249]. Ubiquitination can result in
degradation of native correctly folded proteins for the purpose cellular process control including transcription, signal transduction and development [250]. However, ubiquitination can also degrade unfolded or misfolded proteins in the endoplasmic reticulum (ER) through a process termed Endoplasmic Reticulum Associated Degradation (ERAD). Protein folding is inherently error prone. In addition, many variable stress stimuli such as hypoxia, bacterial infection, repetitive ischemia/reperfusion and altered cellular and systemic stress responses bear on chronic pressure ulcers and may compromise the rate or efficiency of protein folding [251][252].

Proteins are assembled in the endoplasmic reticulum and secreted proteins are subjected to ER quality control [253]. When proteins attain their native conformations they may be directed to their final destination. When proteins do not achieve their native conformations they are subjected to further processing to achieve proper folding. If the proteins do not achieve their native conformation after ER quality control processing then they are subjected to ERAD [254]. ERAD is a complex process and proteosomal degradation can occur with mechanisms outside the ubiquitin pathway, however ubiquitination is the major mechanism associated with degradation of proteins through the proteosome [252].

It is well established that attachment of ubiquitin occurs through a cascade of three enzymes; E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (Ubiquitin E3 Ligase) [252][255]. Activation of ubiquitin by E1 initiates the process. Then the activated ubiquitin is transferred to the E2 and finally the E2 conjugate associates with the E3 ligase and the E2/E3 complex transfers the ubiquitin to a lysine in the protein target [256]. The specific combination of E2 and E3 enzymes determines the chain linkage type [257]. Ubiquitin is the common denominator for proteosomal, lysosomal and autophagosomal degradation [257].
The IPA® core analysis was performed on the whole genome microarray as described. The normal skin microarray and the chronic wound microarray each produced 25 individual, 35 gene networks. Investigation of the core analysis showed that the major network (most significant) for both the normal skin control (Score: 36) and the chronic pressure ulcer (Score: 36) was ubiquitin C centered. Ubiquitin C is an E2 enzyme that has been shown to be a part of evolutionarily conserved E2/E3 complexes [250]. Normal skin and chronic wound networks were 77% similar with 27 identical genes and 8 different genes associated with UBC (Figure 18).

CERCAM (cerebral endothelial cell adhesion molecule), LMF2 (Lipase maturation factor 2), SLC22A17 (solute carrier family 22 member 17), UBAP1 (Ubiquitin associated protein 1), RNF145 (ring finger protein 145), and C8orf76 (chromosome 8 open reading frame 76) were all upregulated in the chronic pressure ulcer as compared to the normal skin control. CLIP3 (CAP-GLY domain containing linker protein 1) and RNF24 (ring finger 24) were the only genes that were not transcriptionally affected, however CLIP3 and RNF24 became associated with Ubiquitin C in network 1 of the chronic pressure ulcer (Table 5) indicating a functional shift involving the association between UBC and these genes. In general, it can be stated that these eight genes are associated with Ubiquitin C (UBC) in the chronic wound and may represent treatment targets or treatment indicators in these currently difficult-healing chronic pressure ulcers.

Six of the eight different genes in the normal skin control (Figure 18) possessed transcriptional differences (Table 6.). Additional gene information can be found in Appendix 2 and 3. Of the eight genes, only NCCRP-1 (non-specific cytotoxic cell receptor protein 1 (zebrafish)) was upregulated in the chronic pressure ulcer. KPRP (keratinocyte proline rich protein) and RILPL1 (Rab interacting lysosomal protein-like 1) did not have significant
expression changes. DEF8 (differentially expressed in FDCP 8 homologue (mouse), THUMPD1 (THUMP domain containing 1), MMGT1 (membrane magnesium transporter 1), BEX4 (Brain expressed X-linked 4), and SECISBP2L (SECIS binding protein-2 like) were all slightly downregulated. These findings imply a loss of normal skin functionality related to these UBC-associated genes.

Figure 18. Network schematic of genes that are associated with UBC in the A) the normal skin network #1 or B) the chronic pressure ulcer network #1. These genes are present in both the normal skin and the chronic pressure ulcer. The 8 genes associated with UBC in the chronic pressure ulcer are not associated with UBC in the normal skin and the 8 genes associated with UBC in the normal skin are not associated with UBC in the chronic pressure ulcer.
Cerebral Endothelial Cell adhesion Molecule (CERCAM) is a gene that is significantly associated with UBC only in the chronic pressure ulcers. This gene is widely transcribed in the nervous and secretory tissues including salivary gland, pancreas, liver and placenta [258]. CERCAM has been shown to be a glycosylated binding partner of Fbox06 [259]. CERCAM is related to CoLGALT1 and CoLGALT2 but does not possess the ability to glycosylate collagen and it possesses an ER localization signal and localizes to the ER like COLGALT1 and COLGALT2 [258]. CERCAM was first described as a cerebral adhesion molecule and bound to CDllb/Cd18 and was suggested to be involved in leukocyte trafficking which is associated with inflammatory processes [260]. It is unknown whether this protein glycosylates different proteins other than collagen [261].

CERCAM was mapped to a genetic locus in the wrinkle free mouse (wrfr), an autosomal recessive mouse mutation that results in extremely tight, thick skin [262]. Interestingly, the wrinkle-free mice die shortly after birth and the phenotype is similar to restrictive dermopathy [262]. Taken together, these observations suggest that CERCAM is associated with a defective skin barrier while facilitating leukocyte trafficking and that these processes are associated with Fbox06 related ubiquitination processes in the chronic pressure ulcers of SCI patients.

CERCAM was found to be upregulated (2.89 Fold, p < 0.001) in the chronic pressure ulcers (Table 5.). Targeted core and stress regulator network analysis demonstrated that CERCAM associated with p53 and CDK2 through UBC and SQSTM1 suggesting that these core regulators may influence the expression of CERCAM and the SQSTM1-p53 association may influence the UBC-CERCAM interaction and influence the stress response and/or survival decision made by the cells.
LMF2 (lipase maturation factor 2) is another gene that, similarly to CERCAM is a glycated FboxO6 binding partner [259]. LMF2 is slightly yet significantly upregulated (1.23 Fold, p< 0.05) in the chronic wound. This further supports a role for FboxO6 in ubiquitin regulation of proteins in the chronic pressure ulcer. FboxO6 is associated with ubiquitination and degradation of glycated proteins in a process termed GERAD (Glycated Endoplasmic Reticulum Associated Degradation) [263]. The significant association of FboxO6O associated genes with UBC may point to the importance of ubiquitination of glycated proteins in the CPU.

LMF2 was originally identified as a potential gene involved in lipid metabolism [264]. LMF2 is 42% homologous to LMF1, however, LMF2 cannot complement or rescue LMF1 deficient cells suggesting a different role for LMF2 [264]. While a physiologically relevant and non-redundant role was suggested for LMF2, there is no further information about this gene available.

Little is known about the function of RNF145 although it was found to structurally cluster closest with RNF139 (TRC8) with both RNF proteins containing multiple membrane domains [265]. RNF145 is a putative human transmembrane RNF protein [266][265]. RNF domains are composed of 40-80 amino acids and contain eight conserved cysteine and histidine residues that chelate two zinc ions to form a cross-brace structure which allows correct folding and function of the RING [266][265]. RNA domains have 3 classifications based on the structure of the finger: C3H4 (RING-HC), C3H2C3 (RING-H2) or C4HC3 (RING-CH or RINGv). RNF 145 possesses the C3HC4 (RING-HC) RNF domain. The RNF domain is a scaffold for binding to E2 enzymes closely with substrate proteins which allows for efficient transfer of ubiquitin to substrate proteins [267].
Ring Finger 145 (RNF145) was found to be significantly upregulated (1.82 Fold, \( p < 0.001 \)) in the chronic pressure ulcers (Table 5.). Humans possess an expanded repertoire of E3 Ligases that are available to accomplish ERAD [268]. RNF 139 was found to be an E3 Ligase that is involved in ERAD of normal and abnormal proteins and intriguingly, this multi-function ligase is targeted by a human CMV virus protein which results in ubiquitination and destruction of MHC I [268]. In a similar fashion, the high structural similarity of RNF145 to RNF139 suggests that this E3 ligase is involved in ERAD within the CPU. RNF 145 function and interactions have not yet been characterized yet RNF 145 upregulation along with UBC association in the chronic pressure ulcer demonstrate that RNF145 may play a role in proteosomal protein degradation. Individual analysis of RNF145 in relation to targeted core and stress regulators associated p53 with RNF145 through UBC. These observations imply that RNF145 may be functionally associated with p53 directed processes within the chronic pressure ulcer.

UBAP1 is another transcriptionally regulated gene (1.53 fold, \( p<0.001 \)), UBAP1 (ubiquitin associated protein 1) is a component of endosomal sorting complex 1 (ESCRT1) and is associated with Vps28, Vps37A and TSG101 [269][270]. The ESCRT system has been well described [271-274]. ESCRTS are multi-protein complexes that function in the biogenesis of multi-vesicular bodies [272]. UBAP1 forms a heterotimeric protein complex that contains multiple ubiquitin binding domains with Vps28, Vps37A and TSG101 allowing ESCRT1 to readily acquire ubiquinated membrane proteins cargos [269].
Figure 19. UBAP1 Related Core Master Transcriptional Regulator Network

Genes above the large box represent members of the UBAP1 containing ESCRT1 system.
ESCRT1 consists of Vps28, Vps37A, TSG101, and UBAP1. Known linkages of ESCRT1 with the main transcriptional regulators are through GCR (glucocorticoid receptor) and ERK1/2.
Genes/histones to the right of the box represent known links to the transcriptional regulators.
p300/CBP provides a linkage that unites all the transcriptional regulators within the large box containing the most significantly associated transcriptional regulators. The dotted line indicates the EGFR regulation by ESCRT1 through UBAP1.
Membrane bound proteins are sorted through multi-vesicular bodies upon binding to ubiquitin. Ubiquitinated proteins are then recognized through a series of ESCRTs which contain multiple ubiquitin binding domains coordinate and complete the formation of intraluminal vesicles.

A salient feature of UBAP1 in this system is the linkage between UBAP1 and EGFR degradation. UBAP1 was shown to be necessary and sufficient to degrade EGFR from the cell membrane via ubiquitin binding and lysosomal degradation [272][272]. EGFR signal transduction is well documented to boost immune and tissue repair abilities of damaged tissue [275-277]. Thus, loss of EGFR and its signal transduction abilities greatly alters the ability of the cells in the wound edge to respond with programs of tissue repair or immunity. EGFR transcription was also affected. EGFR was downregulated (-1.71 Fold, p<0.001)) in the CPU as compared to the normal skin control. Individual analysis of UBAP1 in relation to targeted core and stress regulators demonstrated a relationship with a set of core transcriptional regulators (Figure 4). UBAP-1 demonstrates how ubiquitin integrates proteosomal and lysosomal degradation; signal transduction and transcriptional regulation to obtain a biological response. The upregulation of UBAP-1, association of UBAP-1 with UBC in the chronic pressure ulcers with concomitant downregulation of EGFR transcription indicates transcriptional and possibly ubiquitin/proteosomal downregulation of EGFR in the chronic pressure ulcer.

SLC22A17 is also a gene shown to have significant transcriptional regulation (in the chronic wound analysis. SLC22A17 (Solute Carrier Family 22, Member 17) is a 25kDa protein that belongs to the lipocalin superfamily. SLC22A17 is structured to uptake small lipophilic molecules including hormones, retinoids, arachadonic acid and fatty acids. SLC22A17 will transport its lipocalin 2 (LCN2) ligand either alone or bound to iron [278]. Transport of Lipocalin bound with iron leads to cell survival whereas unbound lipocalin leads to apoptosis.
It was also shown that LCN2 had anti-microbial properties by sequestering iron away from invading bacteria [279].

Previously it was demonstrated that BCR-ABL would influence the expression of LCN2 and SLC22A17. BCR-ABL is a fusion protein that possesses constitutively active ABL tyrosine kinase activity promoting proliferation and survival [281]. BCR-ABL signaling caused a dysregulation of LCN2 and SLC22A17 where LCN2 was upregulated in BCR-ABL containing cells while SLC22A17 was not leading to an apoptotic effect on surrounding cells but allowing survival of transformed BCR-ABL cells. Overexpression of SLC22A17 was shown to induce apoptosis in transformed cell lines indicating that downregulation of SLC22A17 is critical for survival in the presence of LCN2 [282]. Together, these observations indicate that the presence of LCN2 with

In contrast to cells containing the BCR-ABL oncogenes, LCN2 in the chronic wounds was upregulated (2.31 Fold, p<0.001)) in conjunction with a slight upregulation in SLC22A17 (1.22 Fold, P<0.05) (Table 5.). LCN2 was prior observed in wound fluid demonstrating that regulation of SLC22A17 is critical to cell survival [283]. While the cells and cellular mechanisms involved are unclear, upregulation of SLC22A17 has been shown to predispose cells with the SLC22A17 receptor to apoptosis through LCN2 while cells that downregulate SLC22A17 are protected from LCN2-induced cell death [284]. Targeted core and stress regulator network analysis demonstrated that SLC22A17 is associated only with UBC indicating an indirect relationship between SLC22A17, UBC and the core stress regulators and demonstrating the importance of ubiquitin in the regulation of this receptor.

LCN2 has been directly associated with non-healing chronic pressure ulcers [283]. Wound fluid from non-healing chronic venous pressure ulcers were shown to contain persistent
LCN2 levels with concomitant Toll-Like Receptor 2 and 4 activities [283]. Healing wounds demonstrated decreased Toll like receptor activity and LCN2 levels [283]. Since Toll-Like receptors are associated with innate immune responses then this implies that an abnormal innate immune response is related to the multifactorial environment present within the chronic wounds. Since 17β-AED and 17β-AET have been shown to possess innate immunity functionality associated with protection and survival in cells undergoing stress then it follows that these hormones may possess the ability to stimulate an effective innate immune response within the chronic wound [103,351].

RNF24 is an E3- ubiquitin ligase that has been associated with Huntington's disease. RNF24 has a C3-H2-C3 (RING-H2) RNF domain [265], is localized to the Golgi apparatus and was shown to interact with ankyrin-repeats domain(ARD) of TRPC (transient receptor potential channel) channels 1,3,4,5,6 and 7 [285]. RNF24 with its RING-H2 RNF domain was shown to not affect calcium channel-induced expression of RNF24, and did not affect the maturation process of TRPC6 and yet significantly reduced the cell surface expression of TRPC6 suggesting that RNF24 interacts with TRPC channels in the Golgi and affected intracellular trafficking [285]. Therefore, it is possible that UBC can affect cell secretion, contraction, growth, differentiation and apoptosis through the regulation of RNF24.

Individual analysis of individual analysis of RNF24 in relation to targeted core and stress regulators associated p53, CTSD, GYS1, and PI3KC3 with RNF24 through UBC. These genes are well known for directing cell death programs in many different cell types [220][286][287][288][289][245]. Transcription of RNF24 is not significantly different between the CPUs and normal skin yet this E3 ubiquitin ligase becomes associated with UBC in the chronic wound suggesting a function for the UBC-RNF24 association in the chronic wounds.
CLIP3 (CAP-GLY domain containing linker protein 3) is a gene that codes for the CLIP-170-related protein of 59 kDa and this protein is different than other CLIP proteins [290]. CLIP3 is a gene related to apoptotic function and is not transcriptionally affected in the chronic pressure ulcers (Table 5). Recently, CLIP3 in conjunction with cylindromatosis turban tumor syndrome (CYLD) protein was shown to regulate RIP1 ubiquitination and promote caspase-8 activation and apoptosis in the context of TNFα signaling [291]. Upregulation of CLIP3 expression enhanced while CLIP3 silencing inhibited the apoptotic activity of CYLD [291]. It was proposed that CLIP3 in conjunction with CYLD accomplishes de-ubiquitination of RIP1 and subsequent apoptosis [291].

CYLD was, however, downregulated (-2.14 Fold, p<0.001) in the chronic pressure ulcers while the CLIP3 expression change was insignificant. CLIP (cytoplasmic linker protein) proteins are associated with intracellular organization and movement through regulation of microtubules [292]. CLIP-3 differs from other CLIP proteins in that it does not bind to microtubules, is localized to the Golgi, contains a Golgi localization domain that is known to be necessary for addressing cytosolic proteins to the Golgi and is part of the trans-Golgi network [293]. Additionally it was noted that CLIP3 was associated with lipid rafts in HeLa cells and regulates AKT cellular compartmentalization by associating with phospho-AKT [290][294]. Overexpression of CLIP3 was shown to interfere with microtubules and thus CLIP-3 is thought to contain some anti-CLIP function thus perturbing intracellular organization and movement [290]. Thus, CLIP3 in the presence of downregulated CYLD may indirectly perturb the intracellular organization and movement of cells and thus the immune response present in the chronic pressure ulcer edges.
It should be noted that RIP1-mediated, pro-apoptotic signaling may be dependent on cell type and context [291]. Individual analysis of CLIP3 in relation to targeted core and stress regulators demonstrated that CLIP3 is mainly associated with RIP1 and TNFRSF1; however, UBC is associated with p53, CDK2 and cJUN. Thus these regulators may indirectly influence the activity of CLIP3 and may affect the cell death decisions of the cell(s).

NCCRP-1 (Non-specific cytotoxic cell receptor protein-1) was originally cloned from catfish, zebrafish, tilapia, gilthead bream and carp and was predicted to be either a Type II or Type III membrane receptor [295- 300]. Non-specific cytotoxic cells are the equivalent to natural killer cells in the teleost fishes [301]. NCCRP-1, however, was absent on the surface of cells and subsequently was found to contain homology with the F-box only proteins [302]. NCCRP-1 activity was suggested to be responsible for immune functions of nonspecific cytotoxic cells in fish [295]. F-box functions include protein-protein binding of protein substrates in E3 ubiquitin multi-subunit ligases for subsequent ubiquitination by the ubiquitin proteolytic system [303].

The microarray analysis indicated that NCCRP-1 was present in the most significant UBC network of the normal skin control; however, NCCRP-1 was upregulated (2.10 fold, p<.001) while the UBC association was lost in the chronic pressure ulcers, in chronic pressure ulcers (Table 5.). NCCRP-1 has been shown to have 5 paralogues in humans: FBX02, FBX06, FBXO17, FBX027 and FBX044 which function as part of E3 ubiquitin ligase complexes [304]. Sequence alignments of NCCRP-1 and structural interpretations suggested NCCRP-1 to be part of the lectin Type subfamily of Fbox proteins and it was suggested to be renamed as FBXO5O [302]. Fbox proteins have been shown to be present and impart functionality to E3 ubiquitin multi-subunit ligases [305][306][307]. Thus, upregulation of FboxO5O protein (NCCRP-1) and upregulation of FboxO6O-associated proteins and the lack of association of NCCRP-1 with UBC
in the chronic pressure ulcers suggest that the NCCRP-1-UBC association has lost significance in the chronic pressure ulcer. Upregulation of NCCRP-1 may indicate an individual functional role in the dysfunction of the chronic pressure ulcer.

Individual analysis of NCCRP-1 in relation to targeted core and stress regulators indicated that the core regulatory genes associated with NCCRP-1 are p53, CDK2, cJUN and ESR1 with indirect associations between ELAVL1 (discussed below) and UBC (Figure 3). These core transcriptional regulators may underlie the upregulation of NCCRP-1. Furthermore ESR1 is known to influence immune functions and its presence may indicate involvement with cJUN, p53 and ELAVL1 in the transcriptional regulation of NCCRP-1 [308]. The exact role of upregulated NCCRP-1 with the loss of UBC-association in the chronic pressure ulcer is unclear.

NCCRP-1 and MMGT1 are genes present in the individual targeted core and stress regulator networks that contain UBC with a connecting network edge to ELAVL1 (ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1(Hu antigen R)). ELAVL1 is an RNA-binding Protein that can promote RNA stability and influence transcription [309][310]. In stress-treated and untreated cells ELAVL1 was shown to elevate some key target genes including p53 which is part of a cellular transcriptional core regulatory unit [220]. Additionally ELAVL1 has been shown to stabilize many target mRNA's including those that encode stress-response and proliferative proteins including CDKN1A, Cyclins, iNOS, HIF1-α, VEGF, SIRT1, TNFα, BCL-2, and DUSP-1 [311-317]. Heat shock studies showed that transcriptional levels of HuR mRNA did not change in heat shock but rather that HuR protein stability was reduced [318]. Ubiquitin has been associated with ELAVL1 through the following observations: ELAVL1 was ubiquitinated in vitro and CHK2 kinase protected against ELAVL1 loss while proteosome inhibition led to an increase in ELAVL1 abundance [318].
In opposition to heat shock, ELAVL1 is downregulated in the chronic pressure ulcers (-2.4 Fold, p <0.001). This observation demonstrates specificity of ELAVL1 to heat shock responses and suggests that the wound environment is not undergoing a heat shock response. Targeted network analysis demonstrated a UBC association with ELAVL1 to the normal skin control proteins. ELAVL1 was not associated with any of the targeted core and stress regulator analyses of the 8 UBC associated proteins unique to chronic pressure ulcers and the transcriptional downregulation of ELAVL1 in chronic pressure ulcers may indicate its background role of this protein in normal skin.

MMGT1 is a gene that encodes a protein that is located in the Golgi complex and post-Golgi vesicles. MMGT1 is known to transport strontium, iron, cobalt and copper [319]. Originally MMGT1 was thought to contribute to the regulation of magnesium-dependent enzymes that are involved in protein assembly and glycosylation [320]. Later, an integrated mapping strategy identified MMGT1 as part of the mEMC (mammalian Endoplasmic Reticulum Membrane Complex) which was one of six ERAD related functional modules that perform substrate recognition, dislocation, extraction, ubiquitination and proteosomal degradation [321]. The function of the mEMC is currently unknown although MMGT1 is present in the normal skin suggesting that the MMGT1 association with UBC/ELAVL1 is present in the normal skin and may be important for the functioning of normal ion transport and ERAD in the skin [321]. MMGT was slightly downregulated in the CPU (-1.16, p<0.001). Individual analysis of MMGT1 in relation to targeted core and stress regulators demonstrated MMGT1-ELAVL1 and MMGT1-UBC associations. However, MMGT1 is not associated with UBC in the CPU. Together these observations suggest transcriptional rather than UBC regulation of these genes in the chronic wound.
BEX4 is localized to the x-chromosome and is located between BEX1 and BEX2 and is notably expressed uniformly and at high levels and is found in the nucleus and cytoplasm (passive diffusion) in the heart, skeletal muscle, liver and kidney [322]. BEX4 expression in primary cell lines correlated with promoter methylation. It was shown that overexpression of BEX4 induced apoptosis in cancer cell lines [323] and that loss of BEX4 was associated with deregulation of c-Myc activity, cyclin D1 levels resulting in cellular transformation [323]. Thus, these data implicated BEX4 as a tumor regulatory protein [323]. Individual analysis of BEX4 in relation to targeted core and stress regulators demonstrated an association with UBC that was indirectly affected by BRCA-1, CDKN1A, p53 and CDK2. TP53 is the common gene between these networks. BEX4 is downregulated (-1.39, p<0.001) in the chronic pressure as compared to the skin control (Table 5.). Together with the loss of association with UBC this gene also demonstrates transcriptional regulation with concomitant loss of UBC association. Both the presence and role of BEX4 and the BEX4-ELAVL1-UBC associations in both the normal skin control and the chronic pressure ulcers of SCI patients are unknown.

KPRP is a newly identified epidermal protein associated with the upper granular components of the cornified layer of the skin [324]. KPRP was previously identified in a specialized high throughput screen of the transcriptome of importance for epidermal barrier function [325]. Independently, KPRP was suggested to be a marker of stratified epithelia (granular layer) with a potential role in keratinocytic development [324]. This gene was expressed in both the skin control and chronic pressure ulcers in SCI patients (Table 5.). Individual analysis of KPRP in relation to targeted core and stress regulators demonstrated a direct association with CDK2 and indirect associations with p53, cJUN, EGFR, GSK3B, PI3K, HoxB1 through Histone 3 (Figure 2). A protein-protein association of CDK2 and KPRP was
identified in embryonic stem cells [326] and may indicate the involvement of stem cells in the skin. Additionally, it was noted that the expression of HoxB1 is associated with regulation of the retinoblastoma tumor suppressor [227] while association with Histone 3 implies epigenetic regulation. These observations demonstrate that KPRP is intricately associated both directly and indirectly with these key core regulators. It is unknown whether loss of the UBC association in the chronic pressure ulcer adversely affects the UBC-related effects on KPRP present in the normal skin.

THUMP1 was identified through a genetic linkage and T-cell gene expression study [327]. THUMP1 was mapped to a peak linkage within Tsi1 (T-cell secretion of interleukin) and this locus is associated with high expression levels of IL-4 [327]. Thus, THUMP1 (Thump domain containing 1) is an immunity related gene that is transcriptionally downregulated between the normal skin control and the Chronic Pressure Ulcers (Table 5.). This gene was proposed to be a key regulator of in-vitro Th2 differentiation at the Tsi1 locus and was found to be over expressed in BALB/c mice as compared with B6 mice [327]. Interestingly, RNA message stability was proposed as a possible mechanism for the differential expression of THUMP1 between the two mice strains and it was noted that message stability could be due to a deletion of an AU-rich element in the BALB/c mouse which would predispose this message for quick degradation if it were present. Targeted core and stress regulator network analysis demonstrated that THUMP1 is associated only with UBC thus an association with the core transcriptional regulators has not yet been shown. THUMP1 is markedly downregulated (-4.93, p<0.01) in the chronic pressure as compared to the skin control (Table 5.). Together with the loss of association with UBC this gene also demonstrates transcriptional regulation. The role of the UBC-THUMP1 association with immune responses in the CPUs is unknown.
DEF8 (Differentially Expressed in FDCP 8 Homolog) is also, associated with immune functions in the normal skin control. DEF8 was identified as one of 32 genes overexpressed in the Cd14+ unstimulated peripheral blood mononcytic control cells from hyper-immunoglobulin (IgE) syndrome (HIES) patient [328]. HIES is characterized by staphylococcal infection, eczema and high levels of IgE [329][330]. HIES is a multi-syndrome disease and it was suggested that the molecule (s) responsible for the disease would need to affect various biological functions rather than only Th1 or Th2 T-cell responses [328]. Targeted core and stress regulator network analysis demonstrated that DEF8 is associated only with UBC demonstrating that an association between DEF8 and the core transcriptional regulators has not yet been shown. This suggests regulation by UBC rather than stress regulators in the normal skin. DEF8 is downregulated (-1.16, p<0.001) in the chronic pressure as compared to the skin control (Table 5.). Together with the loss of association with UBC this gene also demonstrates transcriptional regulation with concomitant loss of UBC association. The precise role of the UBC-THUMPD1 association with immune responses in the CPUs is unknown.

Nitrosylation is a form of oxidative stress. Transnitrosylation is known to affect 3000 proteins and is well associated with nitric oxide synthases which can result in apoptotic programmed cell death [331] [332]. RILPL1 (Rab interacting lysosomal protein-like 1 is associated with cell survival and cell death and it is known to compete with SIAH (a RING finger E3 Ligase) in binding nitrosylated GAPDH generated from diverse cellular stimuli [333]. S-Nitrosylation was shown to eliminate catalytic activity of GAPDH cause binding of GAPDH to Siah (a RING finger E3 ligase)[334]. Binding of GAPDH to SIAH uncovers a nuclear localization signal located in Siah and causes nuclear translocation of the GAPDH/Siah complex to the nucleus where it binds to p300/CBP and affects acetylation of master transcriptional
regulators including p53 [334]. RILPL1 is a protein that is also S-nitrosylated and was found to competitively bind to Siah and prevent the nuclear translocation of GAPDH. RILPL1 is not transcriptionally regulated in the CPUs (Table 5.) while GAPDH is upregulated (1.93, p<0.001) fold in the CPU. This may indicate an increased sensitivity of the cell toward apoptosis from oxidative stress in the CPU wound edges.

Individual analysis of RILPL1 in relation to targeted core and stress regulators demonstrate an association of RILPL1 with SQSTM1 (p62) showing the significance of the RILPL1 and SQSTM1 association. This association was discovered in the delineation of the response of the toll like receptors to viral infections [335]. Individual stress network analysis demonstrated that RILPL1 is affected indirectly by both NFkB and AP-1 through SQSTM1. RILPL1 is located in the normal skin control and is associated with ubiquitin. This association is lost in the CPU and may indicate a different biological effect of RILPL1.

SECISBP2L (SECIS binding protein 2-like) is involved in selenocyteine incorporation into selenoproteins in humans [336]. Selenoproteins have an essential physiological role in oxidative stress defense [337]. Selenium deficiency, and numerous mutations in selenoproteins and selenoenzymes have been linked to various disorders of the endocrine, central nervous, muscular, cardiovascular, and immune systems in man [338] SBP2-like (SECISBP2L) protein is a homologue of SBP2 [336]. Survey of eukaryotic SECIS binding proteins (SBPs) found that SBP2 and SECISBP2L are paralogues in vertebrates [336]. SECISBP2L is the sole SBP in some invertebrates including sea urchins, sea squirts, and an annelid worm in the genus Capitella [339]. Conservation between SBP2L in vertebrates, invertebrates and mammals suggested role for SBP2L in the post-transcriptional regulation of selenoprotein expression [339].
SECISBP2L and SBP2 were found to be different in their ability to incorporate selenocysteine and SBP2 or SECISBP2L immunoprecipitation experiments showed specific association of selenoprotein mRNAs (GPX1 and GPX4) with both SBP2 and SECISBP2L [336]. The results showed that mammalian cells have at least two selenoprotein mRNP populations and suggested a role for SECISBP2L in the post-transcriptional regulation of selenoprotein expression [336]. SECISBP2L was associated with UBC in the normal skin controls but was transcriptionally downregulated (-1.39 Fold, p<0.001) in the chronic pressure ulcer tissue (Table 5.). Individual analysis of SECISBP2L in relation to targeted core and stress regulators demonstrated an indirect association of SECISBP2L with core stress regulators. Together with the loss of association with UBC this gene also demonstrates transcriptional regulation with concomitant loss of UBC association. The association with UBC in both the normal skin control and the chronic pressure ulcers of SCI patients are unknown.

Summary

Chronic pressure ulcers are notoriously hard to heal. One main problem is the fact that these develop over bony prominences due to pressure, moisture and shear forces [340]. Ulcer formation over the bony prominence then predisposes the affected individual to microbial infection and secondary health problems such as osteomyelitis [341]. Skin substitutes have been evaluated and are sometimes utilized on the legs and feet where they have some success but information is generally lacking and it is known that these are not very effective where bony prominences exist. Additionally, evaluation of CPU in SCI patients has indicated that all phases of wound healing, with the exception of collagen breakdown, are adversely affected in these
patients with hypoxia and inflammation playing central roles in the detrimental, anti-healing environment present in the CPUs of these patients [342].

In this study we have utilized microarray and IPA®Network analysis to probe wound edges in spinal cord injury patients with chronic pressure ulcers and compared these results with results obtained from normal skin controls in the same patients. Whole genome analysis combined with IPA®Network analysis yielded one, comparable network between the chronic pressure ulcers and the normal skin control that was of the highest statistical significance. This network was UBC centered and differed in 8 genes from normal skin (Table 5).

The central position of UBC is critical because ubiquitin is the central common denominator for both the chronic pressure ulcer and the normal skin. Ubiquitination is a post-translational modification that can function in different ways but includes proteosomal, lysosomal and autophagosomal degradation [257]. Ubiquitination can also affect signal transduction and activating/deactivating functions associated with genes [305]. Investigation of the each gene within the normal skin and chronic pressure ulcer networks demonstrated that each gene was associated with a particular biofunction (Table 6).
Table 6: Functions of Genes Associated with UBC in Normal Skin Controls and Chronic Pressure Ulcers of Spinal Cord Injury Patients

<table>
<thead>
<tr>
<th>UBC Related Genes</th>
<th>Function</th>
<th>Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Pressure Ulcers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CERCAM</td>
<td>Abnormal skin barrier function/collagen synthesis Leukocyte transmigration</td>
<td>F-box06/GERAD</td>
</tr>
<tr>
<td>LMF2</td>
<td>Unknown but thought to be involved in Lipid Metabolism/GERAD</td>
<td>F-box06</td>
</tr>
<tr>
<td>CLIP3</td>
<td>1) Anti-CLIP function 2) TNFalpha induced apoptosis by regulating Ubiquitination of RIPK</td>
<td>Apoptosis/Intracellular Organization Disruption</td>
</tr>
<tr>
<td>SLC22A17</td>
<td>Apoptosis</td>
<td>Lipocortin signaling</td>
</tr>
<tr>
<td>UBA1</td>
<td>Endosomal Degradation of EGFR</td>
<td>EGFR signaling</td>
</tr>
<tr>
<td>RNFL24</td>
<td>Regulation of TRPC channels</td>
<td>E3 Ligase</td>
</tr>
<tr>
<td>RNFL415</td>
<td>ERAD within the Chronic Wound</td>
<td>E3 Ligase</td>
</tr>
<tr>
<td>CEOR76</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Normal Skin Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KPRP</td>
<td>Normal Skin Barrier Function in Keratinocytes</td>
<td>Keratinocyte sustainment and development</td>
</tr>
<tr>
<td>NCCRP1</td>
<td>F-box050 activity, innate immunity in the skin and tissue regeneration</td>
<td>F-box05</td>
</tr>
<tr>
<td>THUMPD1</td>
<td>Development of Th1 and Th2 Immune responses</td>
<td>Th1/Th2 responses</td>
</tr>
<tr>
<td>MINGT1</td>
<td>Normal Protein Degradation through ERAD and a component of the mEMC of which the function is currently unknown</td>
<td>ERAD</td>
</tr>
<tr>
<td>DEF8</td>
<td>Immune Response</td>
<td>Tumor Suppression</td>
</tr>
<tr>
<td>BEX4</td>
<td>Associated with tumor suppression. Influences cell directed apoptosis</td>
<td>Oxidative Stress Protection</td>
</tr>
<tr>
<td>RILPL1</td>
<td>Oxidative stress protection</td>
<td>Oxidative Stress Protection</td>
</tr>
<tr>
<td>SEC15EP2L</td>
<td>Oxidative stress protection</td>
<td></td>
</tr>
</tbody>
</table>

The eight genes identified as unique to chronic pressure ulcers represent treatable targets of chronic pressure ulcers. Stress gene related network analysis was utilized to probe the nature of the chronic pressure ulcers for the association of transcriptional regulators known to be directed by androstene hormones. Core transcriptional regulators associated with the androstene hormone directed stress response can be seen underlying the association with UBA1 (Figure
19) which was a gene associated with endosomal trafficking and degradation of membrane proteins essential to wound healing. This demonstrates the link between ubiquitin-proteosomal activity and the core regulators transcriptionally affected by androstene hormones.
The study of the cortical adrenal hormones has been intense over the last 70 years. The discovery that hydrocortisone alleviated symptoms of rheumatoid arthritis was a major breakthrough and resulted in the belief that adrenal cortical steroids had anti-inflammatory properties. Subsequently, the discovery and intense study of \( \beta \) androstene hormones (primarily DHEA, 17\( \alpha \)-AED, 17\( \beta \)-AED and 17\( \beta \)-AET) demonstrated that a plethora of debilitating human diseases could be positively affected through the administration of these \( \beta \) androstene hormones. Variable results obtained through different studies have yielded some skepticism about the utility of these hormones despite large volumes of data supporting the positive effects obtained after administration of these adrenal hormones.

There have been few major insights into the cellular and molecular mechanism of action of these androstene hormones in the last 20 years. The overall rationale of these studies was to further advance the knowledge of the cellular and molecular actions related to these androstene hormones utilizing traditional biological (reporter assays and microarray) and advanced systems biology methods (network analysis) with emphasis placed primarily on 17\( \alpha \)-AED and 17\( \beta \)-AED. This represents the first time ever that this approach has been utilized. Furthermore, an additional goal of these studies was to utilize network analysis to determine if the \( \beta \) androstene hormones might have utility in their treatment of chronic pressure ulcers. Together, these studies test the hypothesis that the \( \beta \) androstene hormones will achieve their cellular and molecular
effects through actions that do not necessarily include the nuclear receptors.

Many studies have shown that application of β-androstene hormones resulted in an interaction between different members of the nuclear receptors (androgen, estrogen receptor alpha and estrogen receptor beta). Despite the ability of the androstene hormones to activate the androgen and estrogen receptors, they have also been shown to elicit their biological effects in cell lines devoid of the androgen and estrogen receptors. This is the first study which compares the action of DHEA and its derivatives directly on the human androgen, estrogen, and glucocorticoid receptors.

The inherent androgenicity and estrogenicity of these hormones has been controversial and biological activity (cell proliferation) has been attributed to androgenicity and estrogenicity of mainly DHEA and 17β-AED. These studies, for the first time, demonstrated the differences between DHEA, 17α-AED, 17β-AED and 17β-AET and demonstrated a decrease of androgenicity and estrogenicity that is on orders less than either testosterone or 17β-estradiol. The initial studies sought to characterize the ability of DHEA, 17α-AED, 17β-AED and 17β-AET to both bind the receptor and activate transcription in a cellular constructs containing human nuclear receptors. Thus, these experiments would confirm prior reports and provide the necessary information on 17α-AED and 17β-AET in relation to 17β-AED, which is known to be a weak binder and activator, of the ESR1 and AR. Additionally, no information was currently available for β androstene hormones binding and activation of the human ESR2.

The overall hypothesis of Chapter 1 was: Androstene Hormones will bind and activate ESR1, ESR2, AR, and GR differently than their known ligands when compared concurrently on whole cell constructs. Once obtained, the androstene hormone activity could then be compared with the known ligands of the ERα, ERβ, AR or GR nuclear receptors. A second aim to this
hypothesis was to determine if DHEA, 17α-AED, 17β-AED and 17β-AET influence binding and activation of the glucocorticoid receptor when the receptor was activated with its known ligand which would demonstrate if androstene hormones would produce anti-glucocorticoid activity at the level of the human receptor.

The androstene hormones directly activated the ERα, ERβ and AR but at levels that were orders of magnitude less than estradiol and testosterone. These studies demonstrated that the β androstene hormones each produced unique, differential interactions and level of activation of these human nuclear receptors indicating that there may be additional factors involved. This result demonstrated that, at the receptor level, the binding and action of these hormones is different than 17β-estradiol and testosterone on the human ERα, ERβ and AR and their activity with these receptors may not be a requirement for their biological activity. Furthermore, these observations support the hypothesis that the biological effects of the androstene hormones are not primarily mediated through interaction with the human ERα, ERβ and AR. Consistent with these observations, androstene hormones have been shown to promulgate their biological effects when the estrogen and androgen receptors are not present. The results were consistent with prior studies and extended the knowledge of the β androstene hormone interactions with the human ERα, ERβ and AR.

Metabolites of DHEA produced intracellularly [343], such as 17β-AED, Androstenedione, testosterone and dihydrotestosterone will cause activation and transactivation of ERα, ERβ and AR [344]. In Chapter 1 studies, DHEA did metabolize into 17β-AED and therefore the activity of DHEA was, at least in part, due to 17β-AED in the nuclear receptor assay cells, however, it was also demonstrated that, in these whole cell assay constructs, 17α-AED, 17β-AED and 17β-AET were not transformed into any metabolites with the ability to bind
and transactivate the human nuclear receptors tested in Chapter 1. The results directly reflected only the ability of 17α-AED, 17β-AED and 17β-AET to interact with these human nuclear receptors.

Activity of 17β-AED has been associated with anti-glucocorticoid activity. It was not known if these interactions occurred at the level of the receptor. Consequently, we pursued the anti-glucocorticoid activity of the androstene hormones. 17α-AED, 17β-AED and 17β-AET did not directly activate the GR and also did not inhibit the GR ligand, dexamethasone. Conversely, there was differential activity of all the receptors, evidenced only at supra-high levels of androstene hormone and dexamethasone, even in the presence of the AR and GR inhibitor: cyproterone. These results extended the knowledge of the human GR receptor and demonstrated in this construct that at very high concentrations of agonist there is an indirect interaction that occurs outside the pocket of the human GR that affects the activation of the human GR. Thus, anti-glucocorticoid activity that is produced by androstene hormones is derived externally to the “active pocket” of the human GR.

No androstene hormone initiated activation of the human GR receptor and there was a differential effect exhibited by DHEA, 17α-AED, 17β-AED and 17β-AET in the presence of dexamethasone. Together, these observations with the differential biological anti-glucocorticoid actions demonstrated by the androstene hormones indicate that the androstene hormones have a differential, indirect effect on the human GR at the receptor level and may further indicate that these hormones have the ability to modulate glucocorticoid activity. Further studies will need to be performed to examine the biological actions produced by androstene hormones that result in anti-glucocorticoid activity.
The androstene hormones are known to produce diametrically opposite biological effects. 17α-AED is known to induce irreversible apoptosis in lymphoma cells (U937) and irreversible autophagy in T98G, U87MG, LN-Z308 and GBM6 Glioblastoma Multiforme cell lines. The chemically identical epimer of 17α-AED, 17β-AED, is known for protection against diverse stressors and promotes cell survival. The main biological effect (irreversible autophagy) of 17α-AED is now known to occur through the ER stress receptor, PERK, and is propagated through eLF2α and CHOP (Gadd153) in Glioblastoma Multiforme cells. Studies ascribed a structural relationship with its biological activity to 17α-AED. It was shown in these studies that the hydroxyl group in the 3β position was dispensable in relation to biological activity but the hydroxyl group at the 17α-position was critical for the biological activity. Thus overall, we demonstrate that the position of the hydroxyl group at the 17 position of 17β-AED, 17α-AED and 17β-AET determines and the hydroxyl group at 7 position influences the biological activity of these β-androstene hormones at the level of the ERα, ERβ, AR and GR receptors.

In chapter 1 we demonstrated that the structure related to the hydroxyl located at positions 7 and 17 resulted in differential activation of a transcriptional reporter in whole cell constructs. 17α-AED and 17β-AED are chemically identical and only differ at the position of the hydroxyl at the 17 position with respect to the cycloperhydrophenanthrene ring of the hormone. Since 17α-AED and 17β-AED are chemically identical and the biological effects of 17α-AED and 17β-AED are opposing and the biological activity 17α-AED and 17β-AED is attributed to the hydroxyl group at the 17 position and because the biological actions associated with the irreversible cell death stimulated by 17α-AED is known, it was hypothesized that 17α-AED of T98G Glioblastoma Multiforme cells would cause the cells to stimulate gene transcription.
resulting in irreversible cell death while $17\beta$-AED treatment would stimulate gene transcription that would result in cell survival.

Thus, one aim of this hypothesis was to identify a transcriptional, cell-directed genetic program stimulated by $17\alpha$-AED that resulted in cell death while at the same time identifying the transcriptional cell-directed genetic program stimulated by $17\beta$-AED that results in cell survival. Since androstene hormones are naturally produced in humans, this set of genes would also represent a core geneset that human cells utilize to either promote survival or death. Thus broad signal transduction microarray that contained 113 genes was performed on T98G Glioblastoma Multiforme cells that were treated with either $17\alpha$-AED or $17\beta$-AED. It was determined that $17\alpha$-AED or $17\beta$-AED regulated 26 of 113 total genes by greater than one magnitude in opposite directions ($17\beta$-AED >5 fold and $17\alpha$-AED <5 Fold). These twenty six genes are presented in Chapter 2 and represent a specific, core set of genes involved in the transcriptional, cell-directed genetic program stimulated by $17\alpha$-AED or $17\beta$-AED that results in cell death or survival respectively.

The identification of the core set of genes regulated in an opposing fashion by $17\alpha$-AED and $17\beta$-AED and aligned with the known biological results demonstrated that a transcriptional, cell-directed program for cell death and survival exists for these epimers through this common geneset. Thus, this led us to hypothesize that network analysis of this geneset could be utilized to elucidate how these genes were associated with each other thus advancing our understanding of the androstene hormone directed program of cellular life and death. The second aim of this study was to further advance the understanding of the known biological action of $17\alpha$-AED and delineate the action of $17\beta$-AED. Together, these observations would advance our current knowledge of how $17\alpha$-AED or $17\beta$-AED influence cell directed life and death decisions.
Network analysis was performed on the 26 member geneset. Network analysis was extremely useful as the network scores could be utilized for 2 important functions. First, networks (and thus gene groupings) could be ranked by most significant to least significant based on the number and associations of the genes (genes included together within networks are termed focus genes) within the network. Second, the gene groups represent associations of how these genes may be organized biologically.

The overall results presented in Chapter 2 demonstrate that 17α-AED directs its actions through PERK, an endoplasmic reticulum stress receptor, while downregulating BRCA1, BCL2, GADD45, CDKN1A and IRF-1. In opposition, 17β-AED stimulated upregulation of BRCA1 and BCL2 with a concomitant upregulation of GADD45, CDKN1A and IRF-1. When upregulated together (Figure 13), this geneset implies that 17β-AED induces a program of DNA repair and growth control. Microarray and Network analysis also implicated the action of WNT1 and its subsequent action on GSK3β as key components to either stimulate or shut down the eukaryotic translation initiation factor eIF2α and upregulate or downregulate the core transcription regulator p53. Furthermore, examination of the networks and the geneset provided evidence that AKT was differentially modulated by these epimers. Examination of the core transcriptional regulators demonstrated that p53 and RB/E2F core transcriptional units were implicated in the survival or cell death associated with 17α-AED or 17β-AED.

Analysis of the genetic mutations that are common to Glioblastoma Multiforme and application to T98G cells demonstrated that mutations exist in components of the CDKN2A/MDM2/p53 axis, the PTEN and NF1 associated Ras/PI3K axis and the CDKN2A/CDK4/RB axis. It could be seen that 17α-AED affected these pathways through downregulation of the geneset while 17β-AED affected these pathways through upregulation of
the geneset. Importantly, $17\alpha$-AED or $17\beta$-AED exerted their biological effects in the T98G Glioblastoma Multiforme cells despite the presence of mutations in the core transcriptional regulators.

Demonstration of the existing common geneset along with the results presented in Chapter 1 supports the idea that $17\alpha$-AED or $17\beta$-AED exert their effects at least, in part, through an opposing biological action which is related to the structural stereochemistry of these two chemically identical hormones. The 26 gene set of common genes that are regulated in opposition by $17\alpha$-AED or $17\beta$-AED exist and many of them are either known transcriptional master regulators or affect those regulators within the geneset. Through the use of $17\alpha$-AED or $17\beta$-AED, T98G Glioblastoma Multiforme cells, microarray and network analysis it was possible to demonstrate that an opposing biological action that includes transcriptional regulation of a set of influential biological regulators exists and is related to the structural stereochemistry of these two chemically identical hormones.

Core transcriptional regulators within the $17\alpha$-AED or $17\beta$-AED common geneset can be associated with cell directed programs of cell cycle control, cell death, angiogenesis inhibition and metastasis or DNA Repair. It could be seen that $17\alpha$-AED achieved its effects through downregulation of core transcriptional regulators and deactivation of translation initiation resulting in cell death. Downregulation of the common geneset could be seen to augment the action of $17\alpha$-AED and thus expands the current knowledge of how this androstene hormone stimulates Glioblastoma Multiforme cells to direct cell death.

In opposition, upregulation of the common geneset delineated how $17\beta$-AED propagated its effects in Glioblastoma Multiforme cells. The upregulation of the common geneset suggests that $17\beta$-AED propagates a cellular program of DNA repair and growth control (Chapter 3).
Finally, these observations along with the observations presented in Chapter 1 further support and strengthen the argument that while androstene hormone activity is influenced by ERα, ERβ, AR and GR these human nuclear receptors are not the primary arbiter of the cellular and molecular activity of 17α-AED, 17β-AED and 17β-AED. Importantly, the 26 member geneset identified how 17α-AED and 17β-AED stimulate cellular life and death decisions even in the presence of genomic mutation and instability.

Chronic pressure ulcers in spinal cord injury patients represent a multi-factorial, hard-to-treat condition that develops over time in patients with limited mobility. The environment of the chronic wound is one of infection, hypoxia, repetitive ischemia/reperfusion, and an altered cellular and stress response. Once a chronic pressure ulcer has formed it becomes a significant health burden both to patients and the healthcare system. These wounds are particularly difficult since spinal cord injury patients have limited mobility and the ulcers usually form from shear pressures located on bony prominences.

Since, microarray and network analysis was useful in delineating the action of 17α-AED or 17β-AED, this technique was again utilized to briefly analyze chronic pressure ulcer wound edges and normal skin. 17α-AED and 17β-AED exert their effects through an ER stress sensing action and their biological effects are promulgated through a common geneset containing master transcriptional regulators. The master transcriptional regulators within the 17α-AED or 17β-AED common geneset can be associated with cell directed programs of cell cycle control, cell death, angiogenesis inhibition and metastasis or DNA Repair. These properties may indicate an application for 17α-AED and/or 17β-AED in the treatment of chronic pressure ulcers.

Thus, we hypothesized that the master transcriptional regulators regulated by 17α-AED or 17β-AED stimulated cells may underlie the altered cellular and systemic responses seen in the
chronic wound environment. The primary aim was to identify the primary significant difference between the chronic wound and the normal skin and then apply targeted network analysis of these genes with the 17α-AED and 17β-AED common geneset that contains master transcriptional regulators. Association of these regulators with the significant differences within the wound indicates an application for androstene hormones.

The analysis revealed that networks were "rewired" in the chronic wound edges probably reflecting the multi-factorial state known to be present in these wounds. For simplicity, a complete review (Chapter 4) was performed only on network number 1 for two main reasons 1) Network 1 is the most statistically significant network. 2) Network 1 contained mainly similar genes between the chronic pressure and the normal skin. The primary observation is that ubiquitin C is the central molecule of network 1 in both the chronic wound and the normal skin.

Review of the genes revealed that eight genes were different between the chronic pressure ulcer and normal skin in network 1. Functional comparison demonstrated that the genes associated with the chronic pressure ulcer do indeed reflect the multi-factorial state of the chronic pressure ulcer while loss of the eight UBC-associated genes in the normal skin implies a loss of skin functionality associated with these genes. CERCAM and LMF2 were associated with abnormal skin barrier function, abnormal collagen synthesis due to lack of glycosylation and leukocyte transmigration. An important observation is that 17β-AED will significantly increase CD11b/CD18 (MAC-1) after radiation [99]. Since CERCAM was shown to bind to CD11b then the implication is that 17β-AED or 17β-AET would promote beneficial immune cell trafficking and processes in the stress altered environment of the chronic pressure ulcer.

CLIP3 and SLC22A17 were related to cellular apoptosis while UBAP1 suggested lysosomal degradation of EGFR which was accompanied by a transcriptional decrease in EGFR t
while RNF24 and RNF145 represent E3 ligases involved in ubiquitination processes. Together, these processes suggest that the ubiquitin-proteosomal system may be the central factor in regulating the altered cellular and stress response of the chronic wound environment. The main observation is the loss of association of UBC with genes that regulate barrier function in, immunity and oxidative stress, in normal skin.

Targeted microarray analysis of the eight different genes demonstrated that p53, CDK2, SQSTM1 and UBC were active in the regulation of CERCAM. UBAP1 demonstrated a more complex interaction with the common geneset and the glucocorticoid receptor, EGR1, NAB2, GADD45 and BRCA1 were implicated in the regulation of EGFR, which is significantly downregulated and known to play a leading role in the cell survival and proliferation seen in normal wound healing. In addition, CLIP3 analysis added cJUN to the list of regulators associated with the eight UBC-associated genes that are present in the chronic pressure ulcer.

Additionally, EGFR was associated with the presence of the glucocorticoid receptor (Figure 19). The glucocorticoid receptor is known to counteract the activity of the EGFR [346]. Furthermore, the glucocorticoid receptor is known to predispose individuals with chronic stress to mental anxiety, immune deficiency, inflammatory dysregulation and impair wound healing [347][348][349][112]. It has been shown prior that androstene hormones can counteract the glucocorticoid receptor [73] and stress-impaired wound healing [112][170]. It is demonstrated here that the androstene hormones interact indirectly and have applicability to the treatment of chronic wounds since it is demonstrated here that the androstene hormones influence the human GR indirectly, influence biological activity of the human GR and a subset of the 26 genes known to be transcriptionally regulated by androstene hormones (core regulators) are associated with the UBC-associated genes unique to the chronic pressure ulcers.
A critical observation is that NCCRP-1, CERCAM, ThumpD1, DEF8, CLIP3, RNF24, SLC22A17, UBAP1 were genes in both the normal and chronic pressure ulcer skin that represent activity of the innate immune system. Thus, NCCRP-1, ThumpD1, and DEF8 loss of association with UBC in the chronic pressure ulcer represents a loss of normal innate immune function while the association of CERCAM, CLIP3, RNF24, SLC22A17 and UBAP1 represent an abnormal innate immune function in non-healing skin. Since abnormal innate immune functions are associated with non-healing pressure ulcers then these genes can be seen as primary targets of therapies targeting chronic pressure ulcers.

Three main observations demonstrate that 17β-AED and 17β-AET may be effective therapeutics in the chronic wound environment. First, these hormones are known to increase effective innate immune processes in the presence of stress demonstrated by modulation of innate immune cytokines and effective cellular responses. Second, these hormones are known to counteract the effects of the glucocorticoid receptor also through the modulation of innate immune cytokines and cellular responses. Third, 17β-AED is now known to initiate a cellular program of DNA repair, growth control and immunity through the modulation of transcriptional regulators.

The androstene hormones are known to be beneficial to stress-related delayed wound healing and provide the means to discover how core transcriptional regulators can themselves be regulated to influence the eight genes uniquely associated with the ubiquitin-proteosomal system in the chronic wounds. Thus, counteracting the action of the GCR receptor and initiating an effective innate immune response may lead to new and effective therapies in the treatment of chronic pressure ulcers. The genes identified in this analysis may represent treatment biomarkers
which can guide treatments aimed at improve healing or the effectiveness of existing therapies such as application of skin substitutes.

Future studies will need to be performed to elucidating the structure-function relationship of the androstene hormones to further expand on what is now known. Elucidation of interactions between the core regulators and UBC will be critical in understanding how the androstene hormones influence broad biological regulatory processes, including protein production (transcription and translation), modification (phosphorylation, acetylation, ubiquitination etc.) and degradation (proteosomal, lysosomal and autophagic). Understanding how the transcriptional, cell-directed programs stimulated by androstene hormones influence broad biological regulatory processes such as innate immunity or counteract the action of glucocorticoids will illuminate how these adrenal cortical steroids achieve their remarkable biological effects and may result in treatments with applicability to many complex, hard-to-treat or currently untreatable human conditions.
REFERENCES


## APPENDIX 4-A

### Normal Skin, Network 1, UBC-associated Gene Information

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Synonym(s)</th>
<th>Entrez Gene Name</th>
<th>Illumina ID/ Human Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPRP</td>
<td>1110001M24Rik, AA589586, C1orf45</td>
<td>Keratinocyte proline-rich protein</td>
<td>ILMN_1780649/ NM_001025231.1 44834</td>
</tr>
<tr>
<td>NCCRP1</td>
<td>1190020J12Rik, FBXO50, Gm163, RGD1305932</td>
<td>non-specific cytotoxic cell receptor protein 1 homolog (zebrafish)</td>
<td>ILMN_1713397/ NM_001001414.1 342897</td>
</tr>
<tr>
<td>THUMPD1</td>
<td>6330575P11Rik</td>
<td>THUMP domain containing 1</td>
<td>ILMN_2108339/ NM_017736.3 55623</td>
</tr>
<tr>
<td>DEF8</td>
<td>AI449518, D8Ertd713e, FLJ20186</td>
<td>differentially expressed in FDCP 8 homolog (mouse)</td>
<td>ILMN_1767509/ NM_207514.1 54849</td>
</tr>
<tr>
<td>MGMT1</td>
<td>9630048L06Rik, BC032271, EMC5, RGD1566339, RP11-274K13.3, TMEM32</td>
<td>membrane magnesium transporter 1</td>
<td>ILMN_1776216/ NM_173470.1 93380</td>
</tr>
</tbody>
</table>
Normal Skin, Network 1, UBC-associated Gene Information (Continued)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Synonym(s)</th>
<th>Entrez Gene Name</th>
<th>Illumina ID/Accession</th>
<th>Human Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>SECISBP2L</td>
<td>3110001I20Rik, A504340, C63001I23, RGD1559930, RP23-367K9.1, SLAN</td>
<td>SECIS binding protein 2-like</td>
<td>ILMN_1784333/ NM_024077.2</td>
<td>9728</td>
</tr>
<tr>
<td>BEX4</td>
<td>BEXL1, FLJ10097, RP23-1A3.7, RP4-635G19.2</td>
<td>Brain Expressed X-Linked 4</td>
<td>ILMN_1804798/ NM_001006937.1</td>
<td>56271</td>
</tr>
<tr>
<td>RILPL1</td>
<td>2900002H16Rik, 6330559I19Rik, GOSPEL, MNCb-2440, RGD1307973, RLP1</td>
<td>Rab interacting lysosomal interacting protein-like 1</td>
<td>ILMN_1805643/ NM_178314.2</td>
<td>353116</td>
</tr>
</tbody>
</table>
## APPENDIX 4B

### Chronic Pressure Ulcer Skin Edge, Network 1, UBC-associated Gene Information

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Synonym</th>
<th>Entrez Gene Name</th>
<th>Illumina ID/Accession</th>
<th>Human Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CERCAM</td>
<td>2600006K01RIK, AL024097, CEECAM1, GLT25D3, RP11-339B21.2, RP23-443G7.3</td>
<td>cerebral endothelial cell adhesion molecule</td>
<td>ILMN_1750563/ NM_016174.3</td>
<td>51148</td>
</tr>
<tr>
<td>LMF2</td>
<td>AI451006, RGD1306274, TMEM153, TMEM112B</td>
<td>lipase maturation factor 2</td>
<td>ILMN_1716056/ NM_033200.1</td>
<td>91289</td>
</tr>
<tr>
<td>CLIP3</td>
<td>1500005P14Rik, AI844915, CLIPR-59, RGD1306245, RSNL1</td>
<td>CAP-GLY domain containing linker protein 3</td>
<td>ILMN_1789733/ NM_015526.1</td>
<td>25999</td>
</tr>
<tr>
<td>SLC22A17</td>
<td>24p3R, 1700094C23Rik, AU041908, AW555662, BOCT, BOIT, hBOIT, Len2 receptor, mBOCT, NGALR2, NGALR3, NGALR, RBOCT</td>
<td>solute carrier family 22, member 17</td>
<td>ILMN_1653200/ NM_016609.3</td>
<td>51310</td>
</tr>
</tbody>
</table>
### Chronic Pressure Ulcer Skin Edge, Network 1, UBC-associated Gene Information (Continued)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Synonym</th>
<th>Entrez Gene Name</th>
<th>Illumina ID/ Accession</th>
<th>Human Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBAP1</td>
<td>2700092A01Rik, NAG20, RP11-571F15.1, RP23-328E6.5, UAP, UBAP</td>
<td>ubiquitin associated protein 1</td>
<td>ILMN_1807596/ NM_016525.3</td>
<td>51271</td>
</tr>
<tr>
<td>RNF24</td>
<td>2810473M14Rik, 4930505A13Rik, AI317164, C86507, D2Ertd504e, GIL, RP23-387C21.6</td>
<td>ring finger protein 24</td>
<td>ILMN_1717809/ NM_007219.2</td>
<td>11237</td>
</tr>
<tr>
<td>RNF145</td>
<td>3732413I11Rik, FLJ31951, RGD1309561, RP23-103H9.1, TMRF1</td>
<td>ring finger protein 145</td>
<td>ILMN_1710906/ NM_144726.1</td>
<td>153830</td>
</tr>
<tr>
<td>C8orf76</td>
<td>9130401M01Rik, AI849328, RGD1310852</td>
<td>chromosome 8 open reading frame 76</td>
<td>ILMN_1742074/ NM_032847.1</td>
<td>84933</td>
</tr>
</tbody>
</table>
## APPENDIX 4C

Table of UBC-associated Genes Shared in Network 1 of Chronic Pressure Ulcer and Normal Skin Edges

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Skin Control*</th>
<th>Chronic Wound*</th>
<th>Fold Change</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANC6</td>
<td>12</td>
<td>332.12</td>
<td>262.22</td>
<td>-1.27</td>
<td>CCCCCTGGGAATGAGTTGCTTGGCTGTTGGC</td>
</tr>
<tr>
<td>ARL16</td>
<td>17</td>
<td>163.32</td>
<td>125.53</td>
<td>-1.30</td>
<td>AGTGAGCTTGAGAAAGAGGAGAACATGAGT</td>
</tr>
<tr>
<td>C1orf53</td>
<td>19</td>
<td>753.89</td>
<td>54.97</td>
<td>-13.71</td>
<td>GACACACAACACCACTACTCATCTACCTGCCC</td>
</tr>
<tr>
<td>C22orf13</td>
<td>22</td>
<td>1011.37</td>
<td>973.79</td>
<td>-1.04</td>
<td>CCAGACGAAGAGCACTGACGATCGGGACT</td>
</tr>
<tr>
<td>C5orf15</td>
<td>5</td>
<td>683.32</td>
<td>676.38</td>
<td>-1.01</td>
<td>AGTCGGAGAAGAGCAGTACTCCATGGTCCC</td>
</tr>
<tr>
<td>CBOrF88</td>
<td>8</td>
<td>736.81</td>
<td>749.92</td>
<td>1.03</td>
<td>GAAACAATATACAGAGGCGGCAAGTGCC</td>
</tr>
<tr>
<td>C9orf10</td>
<td>9</td>
<td>643.96</td>
<td>609.56</td>
<td>-1.06</td>
<td>CAGCTGTGCTGGCTGACCTGATGACGAC</td>
</tr>
<tr>
<td>CMTC7</td>
<td>3</td>
<td>536.49</td>
<td>38.85</td>
<td>0.07</td>
<td>GCCGTGCTTGCCGCTCTGACGATAAAC</td>
</tr>
<tr>
<td>CRLS1</td>
<td>20</td>
<td>456.81</td>
<td>509</td>
<td>1.11</td>
<td>GCTGAAATACAGATGCTCAATGATACAT</td>
</tr>
<tr>
<td>DCAD1</td>
<td>17</td>
<td>656.92</td>
<td>400.71</td>
<td>-1.69</td>
<td>GGTGCGAGCTCTGTGGCGACCTGCCG</td>
</tr>
<tr>
<td>DDX51</td>
<td>12</td>
<td>66.99</td>
<td>277.5</td>
<td>4.23</td>
<td>GAACACACCTGGCGGCGCTCTGTTGAG</td>
</tr>
<tr>
<td>EFHA1</td>
<td>13</td>
<td>344.22</td>
<td>301.43</td>
<td>-1.14</td>
<td>GACACTCTACGTGTCTCATAATGCGCC</td>
</tr>
<tr>
<td>FAM106C1</td>
<td>15</td>
<td>470.03</td>
<td>669.45</td>
<td>1.19</td>
<td>GTACTCTCTGCCATGCGACGACGAT</td>
</tr>
<tr>
<td>FAM210B</td>
<td>20</td>
<td>281.32</td>
<td>264.42</td>
<td>-1.06</td>
<td>GGGAATAAATCTCAGCCCTAATGAGTCG</td>
</tr>
<tr>
<td>FOPNL</td>
<td>16</td>
<td>261.56</td>
<td>244.07</td>
<td>-1.07</td>
<td>GAGTTCTCTGCGAGAAGTGCAGCGGC</td>
</tr>
<tr>
<td>H3F3C</td>
<td>12</td>
<td>1241.02</td>
<td>1401.25</td>
<td>1.13</td>
<td>TCTACATCAGCTAGCCGCTACGTGAGAAG</td>
</tr>
<tr>
<td>MESTL</td>
<td>2</td>
<td>278.06</td>
<td>302.33</td>
<td>1.09</td>
<td>GCTTGGTCGAGATCTAGAGATGCTGTTG</td>
</tr>
<tr>
<td>MFSD10</td>
<td>4</td>
<td>956.9</td>
<td>1225</td>
<td>1.28</td>
<td>CTTCCGAGATGCGCGAGCTCGAGCTAG</td>
</tr>
<tr>
<td>MRPL21</td>
<td>11</td>
<td>826.07</td>
<td>761.72</td>
<td>-1.08</td>
<td>GACCTGGAAGGCTGAGAGAAATG</td>
</tr>
<tr>
<td>PCLR36GL</td>
<td>1</td>
<td>817.54</td>
<td>566.58</td>
<td>-1.44</td>
<td>GACACTCTACATACCATACCCCTGCCC</td>
</tr>
<tr>
<td>RNASEK</td>
<td>17</td>
<td>846.06</td>
<td>1131.88</td>
<td>1.34</td>
<td>TCCTGGTGCCCTCTCCTGAACCT</td>
</tr>
<tr>
<td>SMAF2</td>
<td>1</td>
<td>76.36</td>
<td>62.77</td>
<td>1.20</td>
<td>GGATGCTGGGCTGCAATGCTGGCTG</td>
</tr>
<tr>
<td>TEM183B1</td>
<td>19</td>
<td>516.84</td>
<td>638.27</td>
<td>1.24</td>
<td>GACAATGGAAGGAGTGCGCTGTTT</td>
</tr>
<tr>
<td>TRMT5</td>
<td>14</td>
<td>4.24</td>
<td>3.9</td>
<td>-1.00</td>
<td>GCCCTTATGCTGGCTGCTGATGAGT</td>
</tr>
<tr>
<td>UBC</td>
<td>12</td>
<td>15590.59</td>
<td>13623.68</td>
<td>-1.14</td>
<td>GCTGAAACCTCTGCTGAGAACCCATG</td>
</tr>
<tr>
<td>ZNF430</td>
<td>19</td>
<td>12.70</td>
<td>3.57</td>
<td>-3.22</td>
<td>GAGGAACCTCTAGTGGAAAGATG</td>
</tr>
<tr>
<td>ZNF4549</td>
<td>19</td>
<td>9.95</td>
<td>3.71</td>
<td>-2.68</td>
<td>CCGCTTATGCGCTGTCGACAGAGCG</td>
</tr>
</tbody>
</table>

26 Genes Common to Chronic Pressure Ulcers and Normal Skin Edges
VITA

Thomas Lee Shaak was born in Lebanon Pennsylvania on 29 August 1968. He is an American citizen and graduated from Lebanon High School, PA in 1986. He is currently at the grade of LtCol, United States Air Force. The following are his earned degrees and work experience:

B.S. Degree in Microbiology from Penn State University, 1990
Awarded the McGinnis Award in Molecular Biology while at Penn State
MT(ASCP) Geisinger Medical Center, PA, 1990
MS Health Care Systems, UMDMJ (University of Medicine and Dentistry of NJ), 2003
MS Biomedical Sciences (Environmental Microbiology), MUSC (Medical University of South Carolina), 2005
Air Force Laboratory Officer, Clinical Lab, Omaha, NB, 1997-2000
Air Force Laboratory Officer, Clinical Lab, Charleston, SC, 2000-2005
Department Chief, Immunology, Air Force Epidemiological Laboratory, SA, TX, 2005-2006
Department Chief Microbiology Air Force Epidemiological Laboratory, SA, TX, 2006-2007
Department Chief Information Systems Air Force Epidemiological Laboratory, SA, TX, 2007-2008
Deputy Chief of the Microbiology Department at Armed Forces Institute of Pathology (AFIP), Wash, DC, 2008-2010
Deputy Chief of the AFIP Base Realignment and Closure, Wash, DC, 2008-2010
PhD Integrative Life Sciences, (Biochemistry), Virginia Commonwealth University, Current

Publication:

1) Structural Stereochemistry of Androstene Hormones Determines Interactions with Human Androgen, Estrogen, and Glucocorticoid Receptors. International Journal of Medicinal Chemistry, 2013,