Effect Of Natural Antioxidants On The Level And Activity Of SIRT1 Protein In Cell Line Of Human Prostate Cancer

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The Prostate Gland:

The prostate gland is a part of the male reproductive system that helps make and store seminal fluid.

A typical prostate gland is about 3 cm long and weighs about 20 gr.

The prostate gland is located under the urinary bladder and surrounds part of the urethra, thus prostate diseases often affect urination.
The Prostate Cancer:

Prostate cancer is a disease in which malignant cells form in the tissues of the prostate.

There are 4 stages that are used for prostate cancer:

1. Cancer is found in the prostate only. It can’t be felt and isn’t visible by imaging.
2. Cancer is more advanced than in stage I, but hasn’t spread outside the prostate.
The Prostate Cancer:

Cancer spread to nearby tissues, usually to seminal vesicles.

Cancer metastasize to lymph nodes or to other organs, such as bones and lungs.
Aging And Prostate Cancer:

Prostate cancer is a major age-related malignancy.

This malignancy is rarely seen in men younger than 45 years. The incidence rises rapidly with each decade thereafter.

![Risk of Being Diagnosed with Prostate Cancer](chart)

*Prostate Cancer is most common in men over 50.*

Because the present life expectancy has significantly improved, it is believed that more cases of prostate cancer will be diagnosed in the future. Thus, it will be immensely useful to better understand the molecular mechanism and connection between aging and prostate cancer.
Aging And The Free Radical Theory:

Aging is a progressive deterioration of physiological functions and metabolic processes.

According to the free-radical theory of aging, organisms age because cells accumulate free radicals and ROS over time, which cause oxidative stress.

The oxidative stress causes damage to the cell components, like DNA, proteins and lipids, and is known to be involved in numerous age-related diseases, like cancer.

Sirtuins – The “Aging Proteins”:
The Sirt proteins, SIRT1-SIRT7, are seven mammalian homologs of yeast SIR2, which regulates longevity at *S. cerevisiae*.

The sirtuins are histone NAD\(^+\) -dependent deacetylases:

The acetyl lysine residues of the target protein serve as substrates for sirtuin deacetylation, and NAD is cleaved to nicotinamide and 2’-O-acetyl-ADP-ribose.

*Fu et al, Mol Cell Bio, 2006*

http://www.ous-research.no/home/institute/news/7248
Sirtuins – The “Aging Proteins”:

Sirtuins may underlie the beneficial effects of caloric restriction, the only non-genetic method that consistently increases maximal lifespan in mammals.

In addition, sirtuins post-translationally modulate the function of many cellular histone and non-histone proteins that undergo reversible acetylation-deacetylation cycles, affecting physiological responses that have implications for treating diseases of aging:

Sinclair et al, Biochem, 2007
SIRT1 – Regulator Of Cell Death/Survival:

SIRT1, the best characterized member among the mammalian sirtuins, plays an important role in the regulation of cell death/survival and stress response.

SIRT1 promotes cell survival by inhibiting apoptosis or cellular senescence induced by stresses, including DNA damage and oxidative stress.

In this context, an increasing number of proteins have been identified as substrates of SIRT1, including p53, FOXO transcription factors, repair protein Ku70, etc.
SIRT1 – A Tumor Promoter Or A Tumor Suppressor?

The controversy over whether SIRT1 serves as a tumor promoter (A) or a tumor suppressor (B) hasn’t been completely solved.

It remains possible that SIRT1 plays dual functions in different tissue contexts, depending on the temporal distribution and abundance of different SIRT1 down-stream targets and factors regulate SIRT1.

*Deng, Int. J. Biol. Sci, 2009*
SIRT₁ And Prostate Cancer:

“Role of Sirtuin Histone Deacetylase SIRT₁ in Prostate Cancer”

Ahmad et al, JBC, 2009
SIRT1 and Prostate Cancer:

SIRT1 is over-expressed at the protein level (A) and enzymatic activity (B) in prostate cancer cells compared with normal prostate cells.

PC3, DU145, LNCaP and 22RV1 – human prostate cancer cells
PrEC – normal prostate epithelial cells
My research purpose was to find a molecule that decreases the SIRT1 protein level and inhibits the enzymatic activity in human prostate cancer cells.

My assumption was that SIRT1 inhibition resulted in a significant inhibition in the proliferation of the human prostate cancer cells.
The main components of NAO are Flavonoid glucoside and p-Coumaric acid.

Those derivatives exhibited a significant decrease in the proliferation of prostate cancer cells. (Bakshi et al, FEBS, 2004).
NAO Purification:

1. Homogenization with H$_2$O
2. Ultrafiltration using a 3K-pore-size membrane
3. Centrifugation

NAO supernatant

1. Evaporation at 80$^\circ$C
2. Mixing with acetone
3. Centrifugation
4. Acetone is evaporated at 85$^\circ$C
5. HPLC analysis and separation
6. NMR

Flavonoid glucoside

p-Coumaric acid
Cucurbitacin E glucoside:

*Citrullus colocynthis (L.) Shard*

http://www.seedman.com/image/d8565.jpg

M.W. = 737 gr/mole

Cucurbitacin glucosides might have therapeutic value against breast cancer cells. *(Tannin-Spitz et al, Biochemical Pharmacology, 2007).*
Cucurbitacin E glucoside Purification:

**Citrullus colocynthis (L.) Shard**

1. Drying the fruit core by lyophilizer
2. Grinding the fruit core

**CC powder**

1. Extraction in chloroform/methanol
2. Filtration

**CC extract**

1. Extraction in chloroform/methanol
2. Filtration
3. Dissolving at EtOAc
4. Separating between the cucurbitacin glucosides by silica gel colony
5. NMR

Cucurbitacin E glucoside
The antioxidant activity of 1,3-diCQA and its ability to scavenge ROS indicate the potential use of this compound to treat disorders involving ROS and oxidative damage, such as age-related diseases.
1,3-diCaffeoylQuinic Acid Purification:

Inula viscosa leaves

1. Mixing with H2O
2. Centrifugation
3. Boiling the supernatant
4. Drying by lyophilizer

Inula viscosa powder

1. Extraction in acetonitrile
2. Extraction the precipitate in methanol
3. Analyzing the methanol extract by TLC
4. HPLC
5. NMR

http://www.wildflowers.co.il/hebrew/plant.asp?ID=323
Cyanidin-3-rhamnoglucoside:

![Image of Ficus carica](http://www.ok-ambiente.com/tag/ficus-carica-l/)

**Ficus carica**

In both *in vitro* and *in vivo* experiments, anthocyanins have demonstrated marked ability to reduce cancer cell proliferation and to inhibit tumor formation.

For example, Cyanidin-3-glucoside decreases the *in vitro* invasiveness of human lung cancer line (*Chen et al*, Cancer Lett, 2006).
The Antioxidant Activity Of The Natural Compounds:

The total antioxidant capacity of the antioxidants is measured by the **ABTS decolorization assay**.

Activity is calculated relative to Trolox and expressed as the concentration of sample necessary to give a 50% reduction in the sample absorbance (IC$_{50}$).

![ABTS reaction diagram](http://bio.classes.ucsc.edu/bio100I/EXERCISES/AO%20ASSAY/ABTS%20reaction.jpeg)
Results – ABTS Assay:

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>IC₅₀ (µM) (average ± S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trolox</td>
<td>21.6 ± 0.01</td>
</tr>
<tr>
<td>C3R</td>
<td>2.3 ± 0.02</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>10.4 ± 0.01</td>
</tr>
<tr>
<td>1,3-diCQA</td>
<td>13.9 ± 0.5</td>
</tr>
<tr>
<td>Cucurbitacin E glucoside</td>
<td>134.3 ± 0.02</td>
</tr>
<tr>
<td>NAO</td>
<td>173.6 ± 0.01 µg/µl *</td>
</tr>
</tbody>
</table>

C₃R > Resveratrol > 1,3-diCQA > Trolox > CC

*Bergman et al, Phytochemistry, 2003*
The Effect Of The Natural Compounds On *In Vitro* Deacetylase Activity Of SIRT1 Enzyme

**Results – Recombinant SIRT1 (X6 HIS) Purification:**
Method – SIRT1 Deacetylase Activity Assay – FDL Assay:

The FDL assay is an assay system designed to measure the lysine deacetylase activity of the recombinant human SIRT1.

The assay has 2 steps:
First, the Flour de Lys-SIRT1 Substrate, which comprises a peptide sequence that include the amino acid lysine is incubated with human recombinant SIRT1 together with the cosubstrate NAD+.

Deacetylation of Flour de Lys-SIRT1 sensitizes it so that, in the second step, treatment with the Flour de Lys Developer (Trypsin) produces a fluorophore. The fluorophore is excited with 360 nm light and the emitted light – 460 nm – is detected on a fluorometric plate reader.
Results – Increase Of Deacetylase Activity Of SIRT1 Enzyme By Resveratrol:

**Effect of Resveratrol on deacetylase activity of SIRT1 enzyme**

- **deacetylation reaction**
- **control reaction - w/o SIRT1**

* * p < 0.05 **, p < 0.01
Results – Decrease Of Deacetylase Activity Of SIRT1 Enzyme By NAO:

* p < 0.05  ** p < 0.01
Results – Decrease Of Deacetylase Activity Of SIRT1 Enzyme By Cucurbitacin:

* $p < 0.05$  ** $p < 0.01$
Results – Decrease Of Deacetylase Activity Of SIRT1 Enzyme By 1,3-diCQA:

![Graph showing the effect of 1,3-diCQA on deacetylase activity of SIRT1 enzyme. The y-axis represents deacetylase activity (AFU), and the x-axis represents [1,3-diCQA] (μM). The graph includes bars for different concentrations (w/o, 8.5, 17, 51, 102, and 204 μM) with statistical significance indicated by * (p < 0.05) and ** (p < 0.01).]
Results – Decrease Of Deacetylase Activity Of SIRT1 Enzyme By C3R:

* p < 0.05  ** p < 0.01
Which antioxidant will be chosen?

<table>
<thead>
<tr>
<th>Natural Compound</th>
<th>IC$_{50}$ (µM) (average ± S.E.)</th>
<th>Maximum % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAO</td>
<td>0.83 µg/µl ± 0.06</td>
<td>95% ± 0.01</td>
</tr>
<tr>
<td>Cucurbitacin</td>
<td>13.22 ± 4.18</td>
<td>61% ± 0.01</td>
</tr>
<tr>
<td>C3R</td>
<td>23.12 ± 0.003</td>
<td>54% ± 0.01</td>
</tr>
<tr>
<td>1,3-diCQA</td>
<td>141.23 ± 1.25</td>
<td>61% ± 0.02</td>
</tr>
</tbody>
</table>

Unique natural antioxidants (NAOs) and derived purified components inhibit cell cycle progression by downregulation of ppRb and E2F in human PC3 prostate cancer cells

Shlomo Bakshi, Margalit Bergman, Sara Dovrat, Shlomo Grossman*

Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel
The Effect Of NAO On PC3 Cells Proliferation

Method – XTT Based Cell Proliferation Assay:

The assay is based on the cleavage of the yellow tetra-zolium salt XTT to form an orange formazan dye by metabolic active cells, which means only viable cells.

The formazan dye formed is soluble in aqueous solutions and is directly quantified using an ELISA reader.
Results – Dose And Time Dependent Effect Of NAO On Proliferation Of PC3 Cells:

<table>
<thead>
<tr>
<th>[NAO] (µg/µl)</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>31%</td>
<td>47%</td>
<td>89%</td>
</tr>
<tr>
<td>1.6</td>
<td>68%</td>
<td>88%</td>
<td>93%</td>
</tr>
<tr>
<td>3.2</td>
<td>72%</td>
<td>97%</td>
<td>95%</td>
</tr>
</tbody>
</table>
Is The Effect Of NAO On PC3 Cells Proliferation SIRT1- Dependent?

A. siCONTROL vs siSIRT1

B. Effect of 24h treatment of NAO on proliferation of PC3 cells - siSIRT1 vs siCONTROL

***, p < 0.01
What is the SIRT1-dependent mechanism of NAO?
NAO Decrease The SIRT1 Protein Level At PC3 Cells:

* p < 0.05  ** p < 0.01
NAO Decrease The SIRT1 Deacetylase Activity At PC3 Cells:

<table>
<thead>
<tr>
<th>NAO (μg/μl)</th>
<th>CONTROL</th>
<th>0.8</th>
<th>1.6</th>
<th>3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-p53/p53</td>
<td>1</td>
<td>1.1</td>
<td>1.22</td>
<td>2.13</td>
</tr>
</tbody>
</table>
Summary & Discussion:

1. The natural compounds that we tested are antioxidants.

2. The natural compounds that we tested inhibited the *in vitro* deacetylase activity of SIRT1.

3. NAO has an anti-proliferate effect on PC3 cells.

4. The anti-proliferate effect of NAO on PC3 cells is mediated by SIRT1:
Future Plans:

1. To investigate the possibility that other substrates of SIRT1 are involved in NAO effect on PC3 proliferation.

2. To study the effect of NAO on in vivo model of prostate cancer, and if this effect is SIRT1-dependent.

3. To compare the efficiency of known SIRT1 inhibitors to NAO as anti-cancer agents.

4. To examine if NAO has anti-cancer effect on other types of cancer, and if this effect is SIRT1-dependent.

5. To test the NAO effect on other sirtuins, including mechanisms.
Thank you for your listening 😊

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The Effect Of The Natural Compounds On In Vitro Deacetylase Activity Of SIRT1 Enzyme

Method – Purification Of 6*HIS-tagged SIRT1 From E.Coli:

A. Protein Expression:
Adding 1 mM IPTG to overnight culture of BL21 with SIRT1 plasmid (pHEX).

B. Sample Preparation to isolate Native Proteins:
Resuspending the cell pellet in 20 mM Tris/HCl (pH = 8), 200 mM NaCl.
Breaking the BL21 by high pressure. Centrifuging the cell extract. The supernatant is the clarified sample.

C. Batch – Flow Column Purification:
Wash Buffer: 20 mM Tris/HCl (pH = 8), 300 mM NaCl, 10 mM Imidazole.
Elution Buffer: 20 mM Tris/HCl (pH = 8), 300 mM NaCl, 200 mM Imidazole.

D. Exchange Buffer – 3,000 Nominal Molecular Weight Limit
Immunoprecipitation and SIRT1 Enzyme Activity Assay—For immunoprecipitation of SIRT1 protein, the lysates containing 500 μg of total protein were incubated with rabbit anti-SIRT1 antibody (Abcam) overnight at 4 °C with constant rotation. The specific antibody-antigen complex was collected by precipitation with Protein A-agarose beads (Pierce) for 2.5 h at 4 °C with constant rotation. SIRT1 activity was determined in immunoprecipitates from cells using the SIRT1 fluorimetric drug discovery kit (AK-555; Biomol) as per the vendor’s protocol.
Results – NAO Has Dose-Dependent Anti-proliferate Effect On PC3 Cells:

**Effect of 72 hours treatment of NAO on proliferation of PC3 cells**

*Cell Proliferation (relative units)*

- **w/o**
- 0.8
- 1.6
- 3.2

[NAO] (µg/µl)

***, p < 0.01**
Method – siRNA Gene Silencing:

SIRT1 siRNA:

Sense: 5'-GAA GUA CAA ACU UCU AGG A dTdT-3'
Anti-Sense: 5'-UCC UAG AAG UUU GUA CUU C dTdT-3'
Similar Researchs:

**A Novel Chalcone Polyphenol Inhibits the Deacetylase Activity of SIRT1 and Cell Growth in HEK293T Cells**

Tomoaki Kahyo¹,*, Shuji Ichikawa², Takahiro Hatanaka¹, Maki K. Yamada¹, and Mitsutoshi Setou¹,³

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**Inhibition of SIRT1 by a small molecule induces apoptosis in breast cancer cells.**


Institute of Life Sciences, University of Hyderabad Campus, Hyderabad, AP 500046, India. arunasreemk@ilsresearch.org

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**Melatonin, a novel Sirt1 inhibitor, imparts antiproliferative effects against prostate cancer in vitro in culture and in vivo in TRAMP model.**

Jung-Hynes B, Schmit TL, Reagan-Shaw SR, Siddiqui IA, Mukhtar H, Ahmad N.
Resveratrol:

Red Grapes
http://www.bobpowell.org/

M.W. = 228 gr/mole


Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan.


BIOMOL Research Laboratories, Inc., 5120 Butler Pike, Plymouth Meeting, Pennsylvania 19462, USA.