Diabetic Retinopathy II

Keith Korthals

September 22, 2005

Proteasome Definition

- A structure inside cells where damaged or unneeded proteins are broken down into 7 to 9 aminoacid chains or polypeptides. Barrel formed structures made of protein which requires ATP to function.
- Digests ubiquitinated proteins usually within cells.
- Ubiquitinated (tagged proteins). Ubiquitin is a protein found in almost all cells. Called the “kiss of death” protein. Named because it is ubiquitous (widely present).

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COMMUNICATIONS

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Mitochondrial Targeting of Selective Electron Scavengers: Synthesis and Biological Analysis of Hemigramicidin−TEMPO Conjugates

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The chemistry and biology of mitochondria, in particular, the effects of intracellular reactive oxygen species (ROS, superoxide radicals and H_2O_2) that are byproducts of the oxidative phosphorylation cascade, is under intense study.[1] Cellular injury, aging, and death, as well as suspended animation, neuro-, and cardioprotection are influenced by events in the mitochondrial membrane that lead to an imbalance in ATP production and O_2 consumption.[2] Recently, dysregulated electron transport and generation of ROS were linked to a mitochondria-specific phospholipid, cardiolipin (CL), and involvement of CL oxidation products in apoptosis.[3] Nitroxide radicals prevent the formation of ROS, particularly superoxide, due to their reduction by the mitochondrial electron transport to hydroxylamine radical scavengers.[4] Nitroxides also exert superoxide dismutase and catalase activities,[5] thus offering additional protective

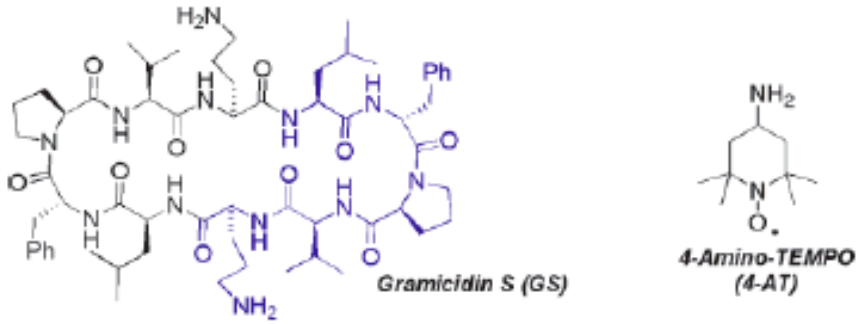
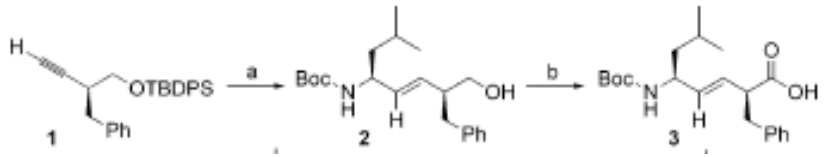


Figure 1. Gramicidin S (targeting sequence in blue) and 4-AT.

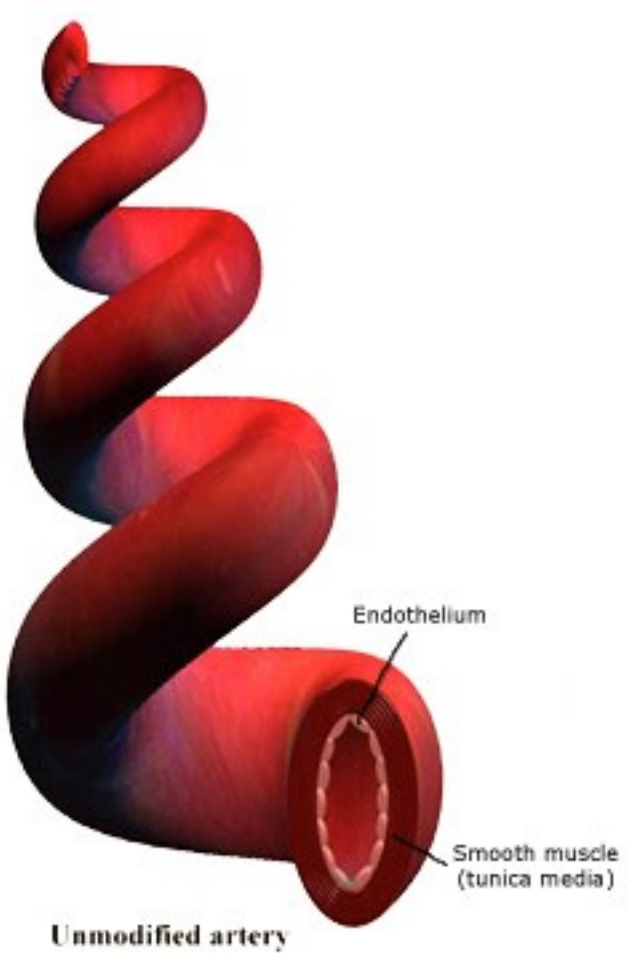
Scheme 1. Synthesis of Peptide Conjugates[a]



Wipf, P.; Xiao, J.; Jiang, J.; Belikova, N.; Tyurin, V.; Fink, M.; Kagan, V. *J. Am. Chem. Soc.* **2005**, *127*, 12460 - 12461.

Diabetes

- Blood sugar levels are regulated by insulin and glucagon
- Normal level 80 to 130 mg/dl. Serious problems at or above 250 mg/dl.
- Some cells can not regulate the sugar level within them. Endothelial cells are one.
- Endothelial cells make up the inside of blood vessels



Diabetic Retinopathy Quick Definition

- Leading cause of blindness
- Diabetes damages the blood vessels in the retina of the eye
- **Mild Nonproliferative Retinopathy.** At this earliest stage, small areas of balloon-like swelling in the retina's tiny blood vessels occur.
- **Moderate Nonproliferative Retinopathy.** As the disease progresses, some blood vessels that nourish the retina are blocked.
- **Severe Nonproliferative Retinopathy.** Many more blood vessels are blocked, depriving several areas of the retina with their blood supply. These areas of the retina send signals to the body to grow new blood vessels for nourishment.

Current Clinical Trials Diabetic Retinopathy

- Steroid slow release implants (goes after swelling). Thought to reduce swelling and prevent the generation of VEGF. Statistically significant improvement in vision (two lines on eye chart).
- Macugen (Eyetech Pharm.) no results (angiogensis)
- Lucentis (antibody fragment) (angiogensis)
- Protein Kinase C Inhibitor (ruboxistaurin, Eli Lilly Co.) angiogensis indirectly - this resulting in moderate effects: it did not show a significant effect on the progression of diabetic retinopathy however did show some prevention of vision loss.
- Although significant beneficial effects nothing halts the progress of the disease.

Problems with the Angiogensis Approach

- The cell death of blood vessels triggers a signal for blood vessel growth in and on the retina.
- Let us assume that you block the blood vessel growth.
- Then you still have the problem of blood vessel cell death to deal with and subsequent retina degeneration.

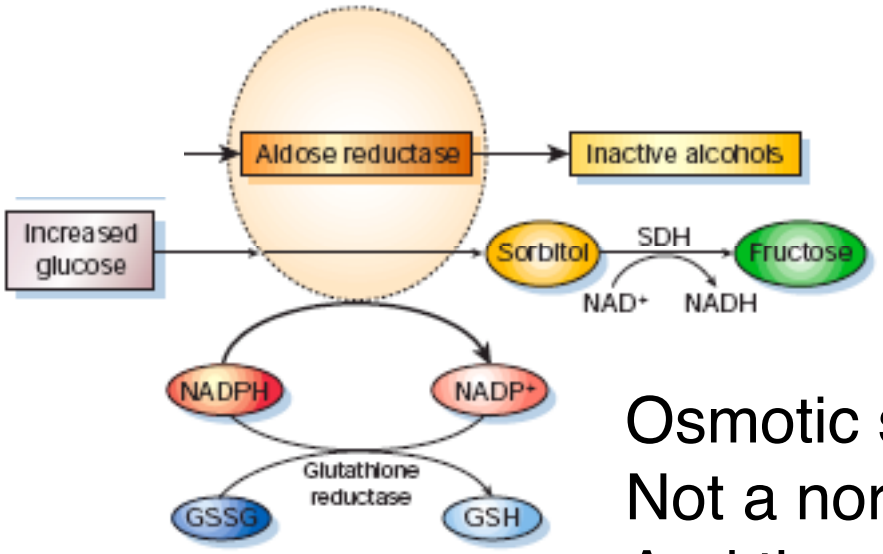
Several Mechanism of Damage

- Increased polyol pathway flux
- Increased advanced glycation end-product (AGE) formation
- Activation of protein kinase C (PKC)
- Increased hexosamine pathway flux

Nishikawa, T.; Edelstein, D.; Xue, L.D.; Yamagishi, S.I.; Matsumura, T.; Kaneda, Y.; Yorek, M.; Beebe, D.; Oates, P.; Hammes, H.P.; Giardino, I.; Brownlee, M. *Nature*, **2000**, 404, 787 - 790.
Brownlee, M. *Nature*, **2001**, 414, 813 - 820.

Increased polyol pathway

- At normal concentration of glucose this pathway is a very small percentage of converted glucose
- At elevated concentration of glucose, it is 11 to 33 % of total glucose converted.



Osmotic stress to cell
Not a normal pathway
And the cell wastes energy

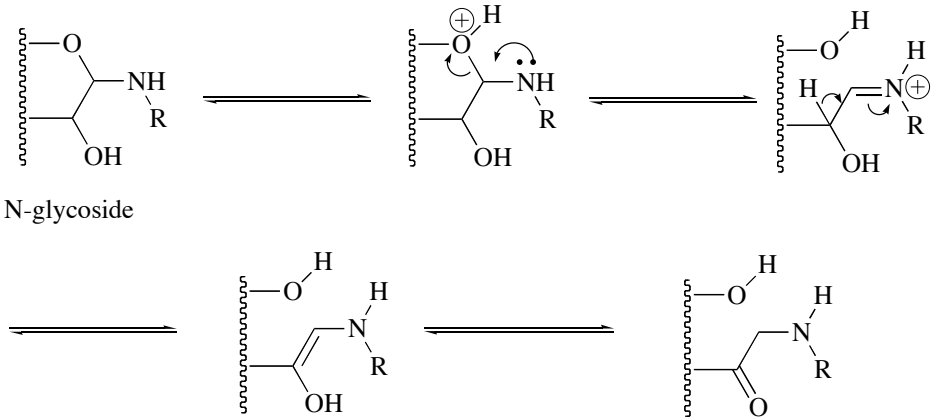
Advanced Glycation End-Product (AGE)

- Proteins that are derived from the oxidation of glucose to glyoxal. Glyoxal react with amino functionality of a protein.
- AGE's are secreted by the cell which activate receptor sites on other cells (macrophage) that activates transcription factors such as NF-kB. Different part of the DNA is converted to RNA and subsequently to a protein.
- Promotes swelling (heart disease) and NF-kB turns on genes to prevent cell death (cancer).

Al-Abed, Y.; Bucala, R. *Bioconjugate Chem.* **2000**, *11*, 39 - 45.

Origin of AGE's

Amadori Reaction / Rearrangement



❖Not enzyme catalyzed. Resulting compounds contribute to aging.

❖Exact chemical elucidation has remained elusive.

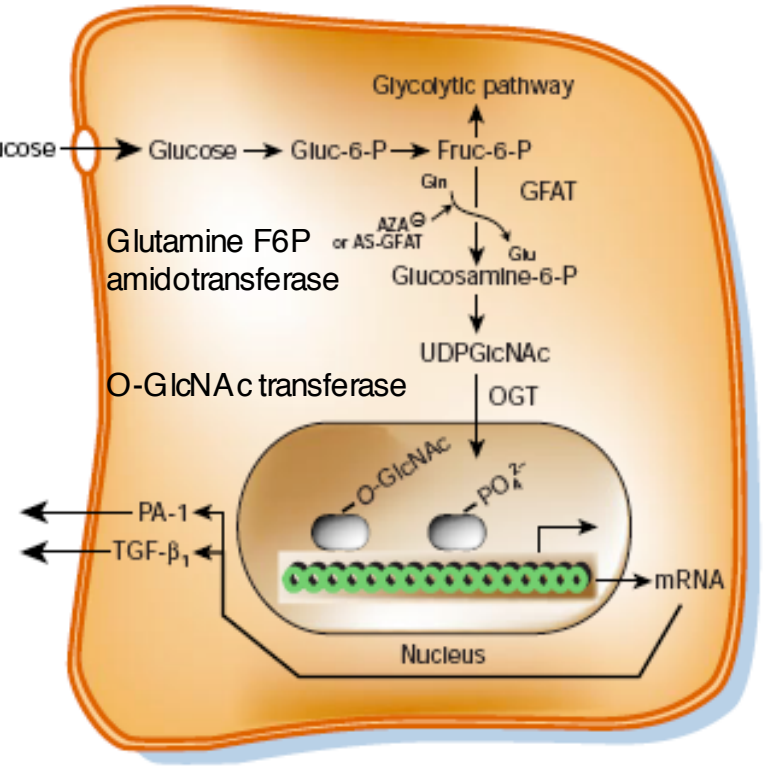
Al-Abed, Y.; Bucala, R. *Bioconjugate Chem.* **2000**, *11*, 39 - 45.

Activation of protein kinase C (PKC)

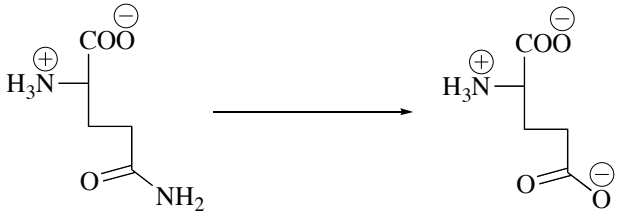
- Eleven similar enzymes
- Transfers a phoshate from ATP to other enzymes. Phosphorylation of tyrosine residues.
- Activation of this enzyme by DAG (diacylglycerol) which is made by a known mechanism from pyruvate.
- DAG increases because its synthesis is pushed forward by the large excess of glucose.
- Activation of protein kinase C leads to abnormal blood flow.
- Stimulates the formation of VEGF.

Hexosamine pathway

- The diversion of fructose-6-phosphate
- Glucosamine-6-P which was diverted from fructose-6-phosphate
- Results in the transcription of the gene for PAI-1. Promotes vascular smooth muscle cells.
- (Plasminogen Activator Inhibitor-1). The mechanism of increased gene transcription is not known.
- TGF-b is a transforming growth factor which is not linked to angiogensis (currently).

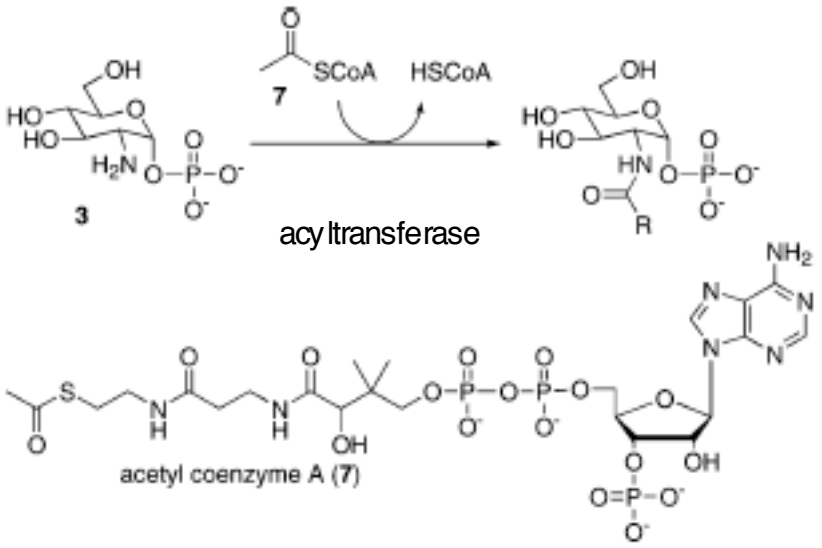


Gln = glutamine
Glu = glutamate

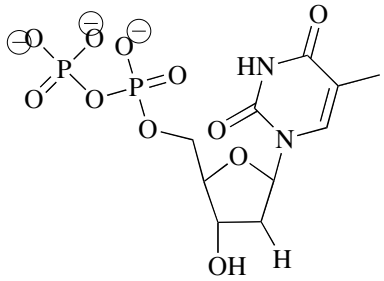


Du, X.; Edelstein, D.; Rossetti, L.; Fantus, I.; Goldberg, H.; Ziyadeh, F.; Wu, J.; Brownlee, M. *PNAS*, **2000**, *97* (22), 12222 - 12226.

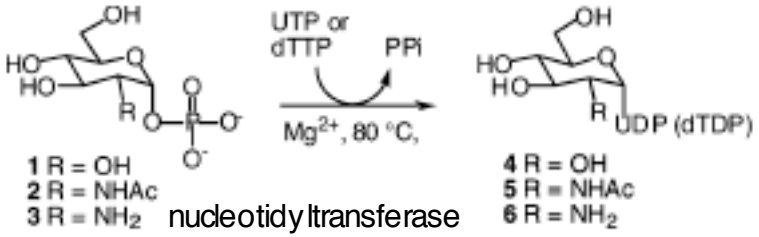
UDPGlcNAc



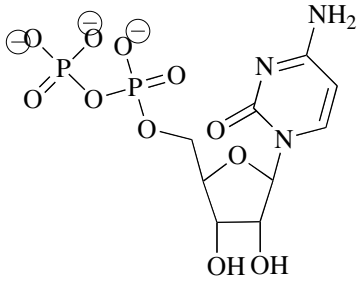
dTTP =



Thymidylate or
deoxythymidinediphospate



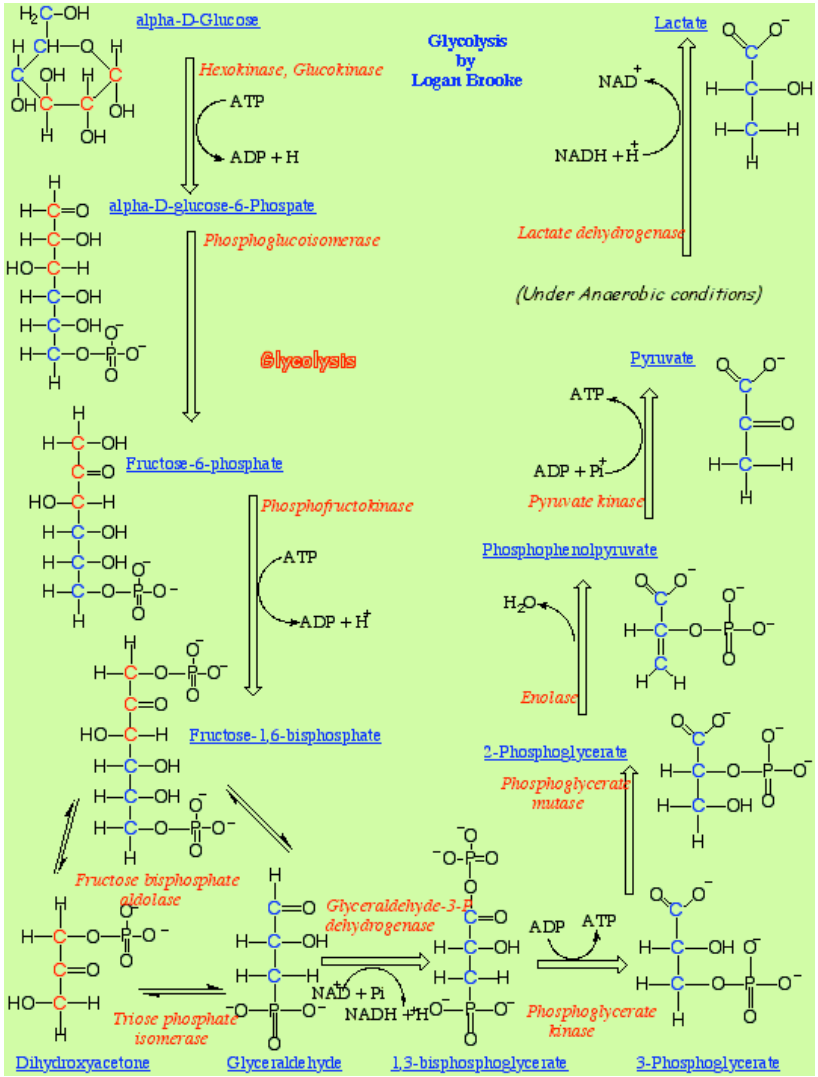
UDP =



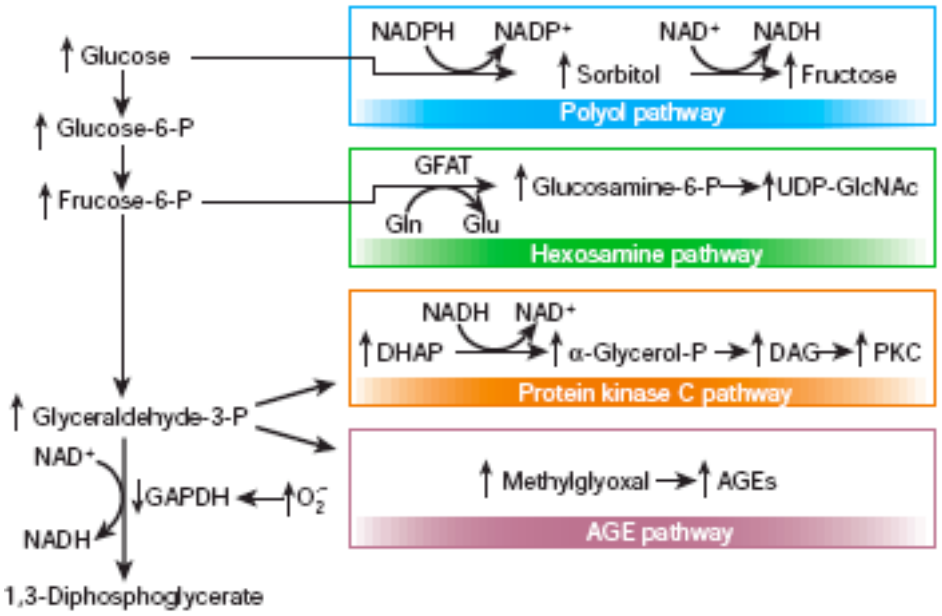
Uridine-5'-diphosphate

Mizanur, R.; Jaipuri, F.; Pohl, N. *J. Am. Chem. Soc.* **2005**, *127*, 837 - 837.

Damaging Pathway



Previously no link between these mechanisms and that link is activated oxygen species.

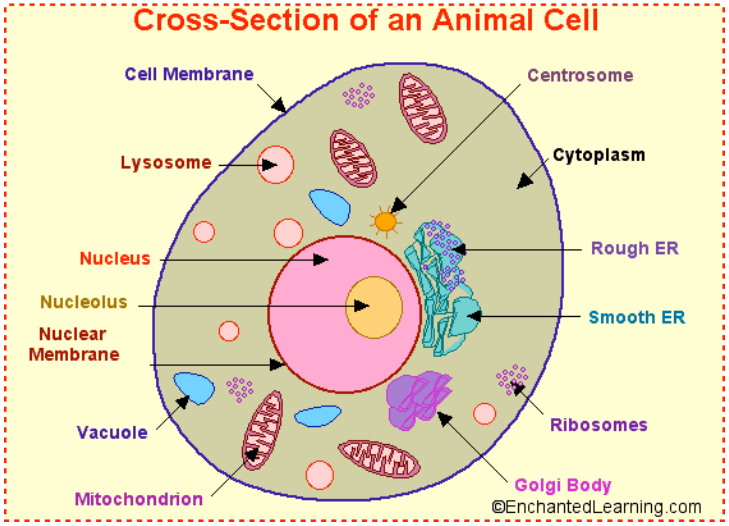


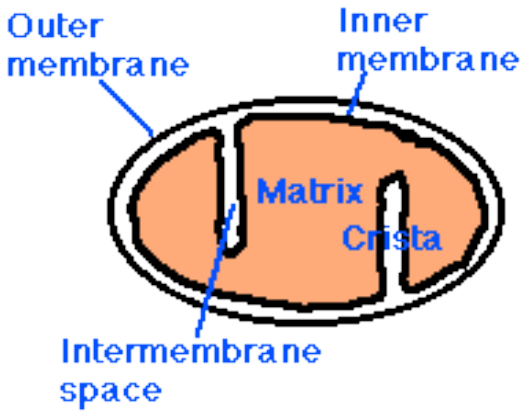
In cytoplasm

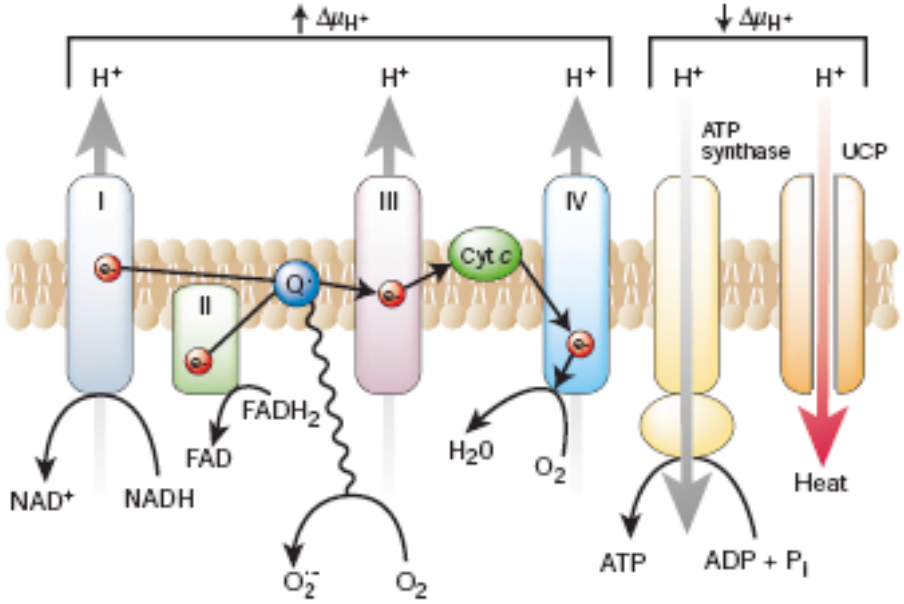
Nishikawa, T.; Edelstein, D.; Xue, L.D.; Yamagishi, S.I.; Matsumura, T.; Kaneda, Y.; Yorek, M.; Beebe, D.; Oates, P.; Hammes, H.P.; Giardino, I.; Brownlee, M. *Nature*, **2000**, 404, 787 - 790.
Brownlee, M. *Nature*, **2001**, 414, 813 - 820.

Damaging Pathway

Inside the Mitochondria







Electron Transport
Complex I ubiquinone oxidoreductase
Complex II succinate-ubiquinone oxidoreductase
Complex III ubiquinol-cytochrome c oxidoreductase
Complex IV cytochrome c oxidase

Nishikawa, T.; Edelstein, D.; Xue, L.D.; Yamagishi, S.I.; Matsumura, T.; Kaneda, Y.; Yorek, M.; Beebe, D.; Oates, P.; Hammes, H.P.; Giardino, I.; Brownlee, M. *Nature*, **2000**, 404, 787 - 790.
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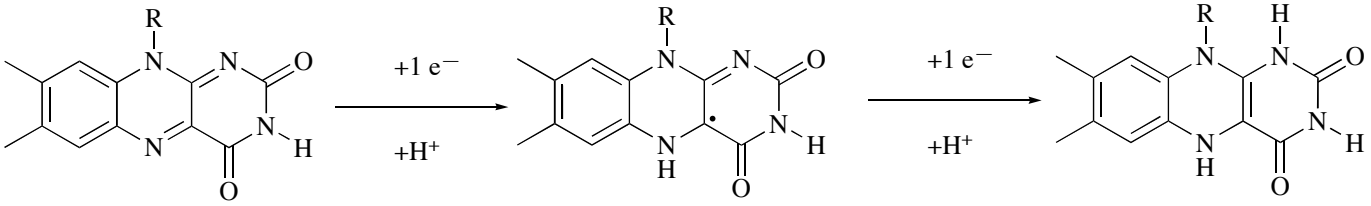
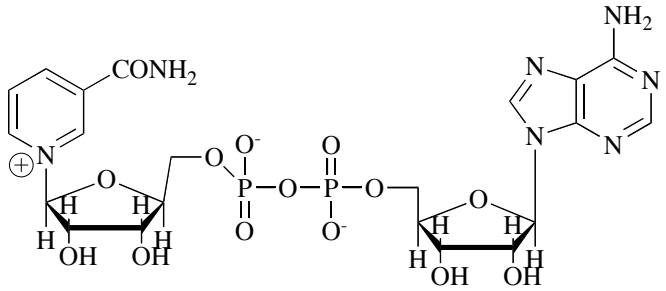
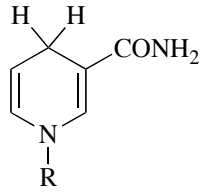
FAD flavin adenine dinucleotide



NAD^+ Nicotinamide adenine dinucleotide

NADH





Problems

- What happens when all this excess glucose is pushed through cell respiration machinery?
- Does ATP accumulate and does it start pushing forward phosphorylation processes?
- Phosphorylation is an important regulation process in cells; it turns on and/or off processes.
- By adding a phosphate the enzyme is either activated or deactivated toward reaction.

Targeting Toward ROS

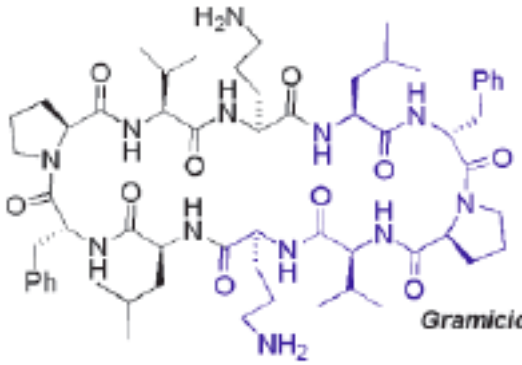
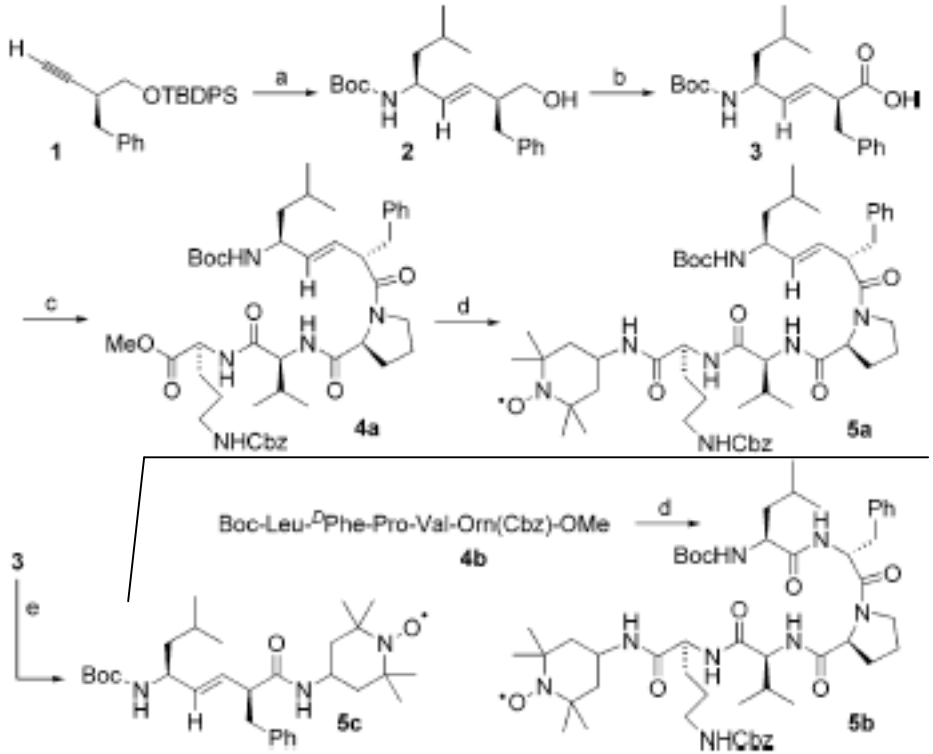


Figure 1. Gramicidin S (targeting sequence in blue) and

Membrane active antibiotic, most of the activity associated with the fragment in blue.

Scheme 1. Synthesis of Peptide Conjugates[a]



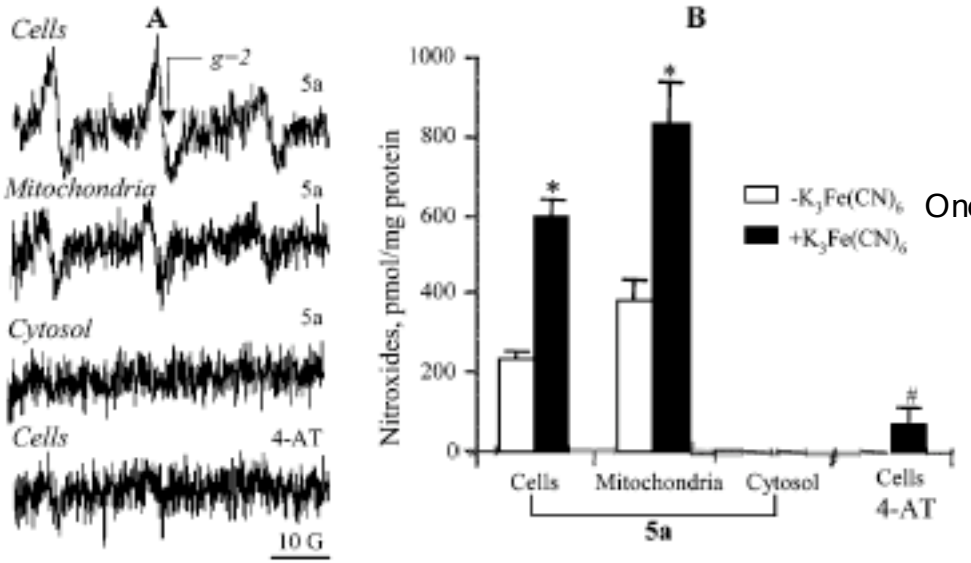
(a) (ii) Real yield of isolated diastereomer was 39 %, and other diastereomer was 39 %, 16 % of a mixture. Compound **4b** was a literature compound.

1:1 dr

[a] Conditions: (a) (i) Cp_2ZrHCl, Me_2Zn, *N*-Boc-isovaleraldimine, then TBAF, 74%; (ii) Ac_2O, TEA, DMAP, 94%; (iii) K_2CO_3, MeOH, quant.; (b) (i) Dess–Martin periodinane; (ii) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene; (c) H-Pro-Val-Orn(Cbz)-OMe, EDC, HOBt, DMAP, 94% from **2**; (d) (i) 1 N NaOH; (ii) 4-AT, EDC, HOBt, DMAP; **5a**, 99%; **5b**, 99%; (e) 4-AT, EDC, DMAP, 91%.

Wipf, P.; Xiao, J.; Jiang, J.; Belikova, N.; Tyurin, V.; Fink, M.; Kagan, V. *J. Am. Chem. Soc.* **2005**, *127*, 12460 - 12461. Wipf, P.; Kendall, C.; Stephenson, C. R. *J. Am. Chem. Soc.* **2003**, *125*, 761 - 768.

Uptake of Compound



One electron-oxidant

Figure 2. EPR-based analysis of integration and reduction of nitroxide GS−peptidyl conjugates in MECs. Cells (10 million/mL) were incubated with 10 μM of 4-AT or **5a** for 15 min. Recovered nitroxide radicals in whole cells, mitochondria, or cytosol fractions were resuspended in PBS in the presence or absence of 2 mM $K_3Fe(CN)_6$ (JEOL-RE1X EPR spectrometer under the following conditions: 3350 G center field; 25 G scan range; 0.79 G field modulation, 20 mW microwave power; 0.1 s time constant; 4 min scan time). (A) Representative EPR spectra of **5a** in different fractions of MECs in the presence of $K_3Fe(CN)_6$. (B) Assessment of integrated nitroxides ($n = 3$); $*p < 0.01$ vs $K_3Fe(CN)_6$; $\#p < 0.01$ vs **5a** under the same conditions.

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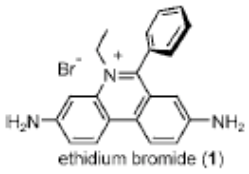
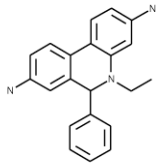
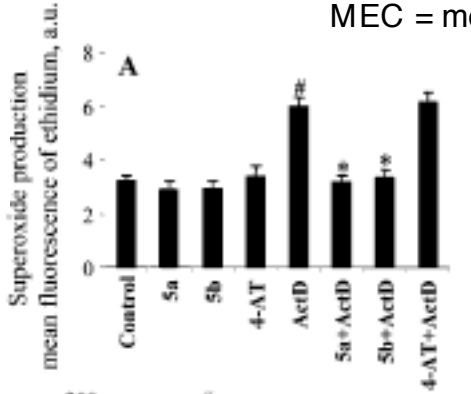
Results of Inhibitor

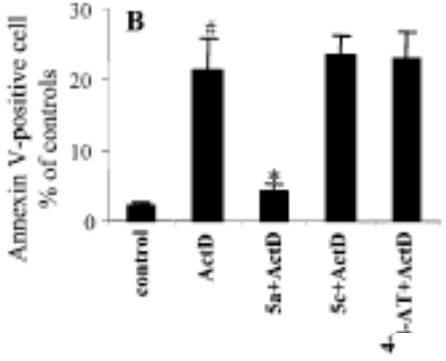
MEC = mouse embryonic cells



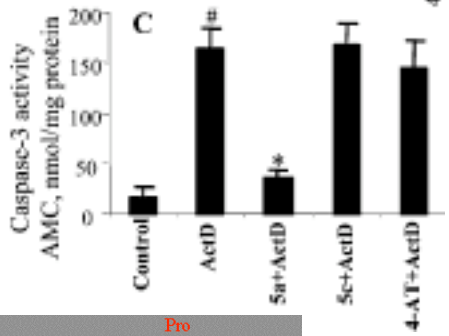
ethidium bromide (1)

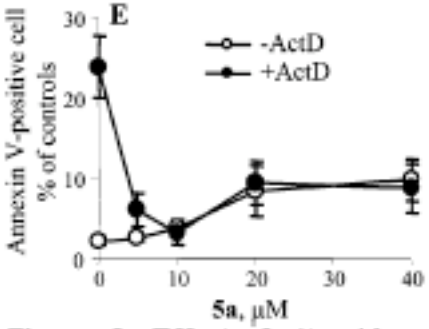


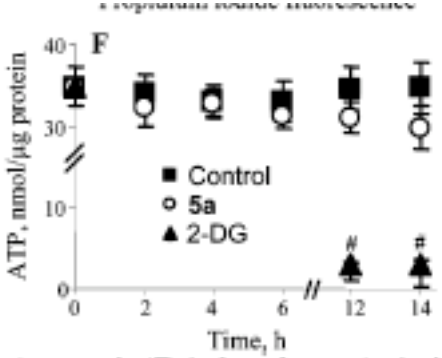




4-AT = 4-amino-TEMP







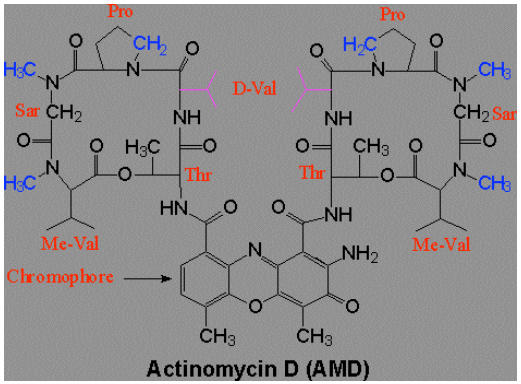


Figure 3. Effect of nitroxide conjugates on ActD-induced apoptosis in MECs. Cells were pretreated with 10 μM 4-AT, **5a**, **5b**, or **5c** for 1 h, then incubated with ActD (100 ng/mL). (A) Superoxide production: mean fluorescence intensity from 10 000 cells. (B) PS externalization. (C) Caspase-3 activation. (D) DNA fragmentation. (E) PS externalization at different concentrations of **5a**. (F) ATP levels in MECs in the presence or absence of **5a** or 2-deoxyglucose (2-DG), as a positive control. Data are means ±SD ($n = 3$), #$p < 0.01$ vs control, *$p < 0.01$ vs ActD-treated cells.

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Apoptosis Indicators

- Annexin V-means phosphatidylserine located on the outer cell wall of cells.
- Caspase activation - cytochrome c complex detected by an assay.