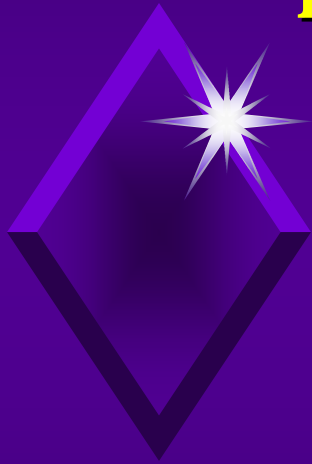
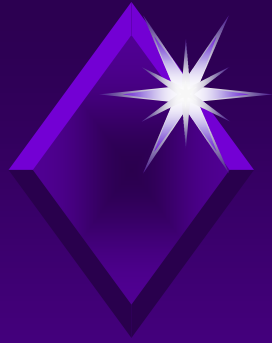


**Designing new agonists and antagonists of
glycoprotein hormones using site-directed
mutagenesis and gene transfer**



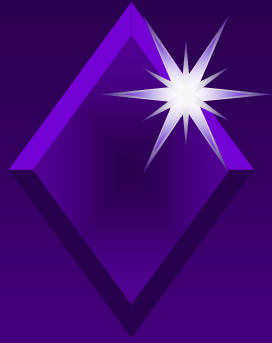
Dr. Fuad Fares

November 8, 2004



Recombinant Proteins

- ◆ Gene Cloning
- ◆ Transfection into Eukaryotic or Prokaryotic cells
- ◆ Purification



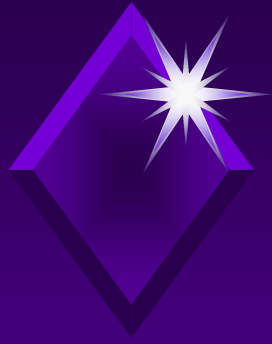
Gene Modifications

- ◆ Site - Directed Mutagenesis

- ◆ Gene Fusion



Development of new agonists
& antagonists

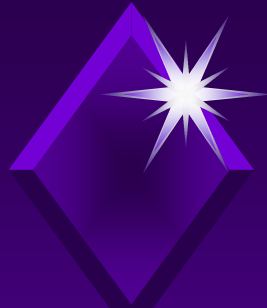


Structure – Function Glycoprotein Hormones

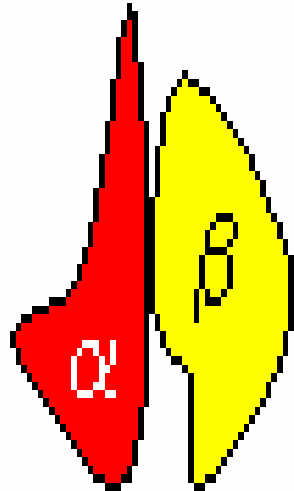
- Site – Directed Mutagenesis
& gene fusion



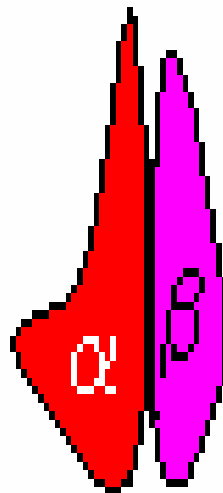
Development of Agonists & Antagonists



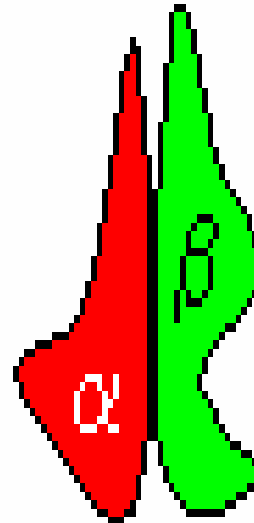
Glycoprotein Hormone Family



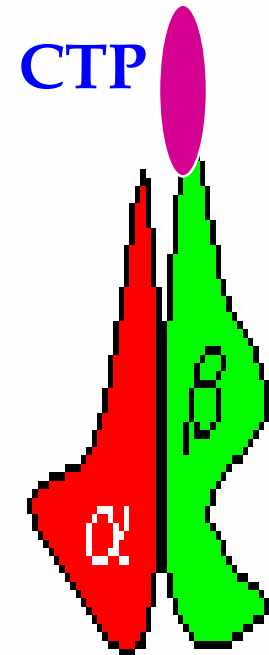
TSH



FSH



LH

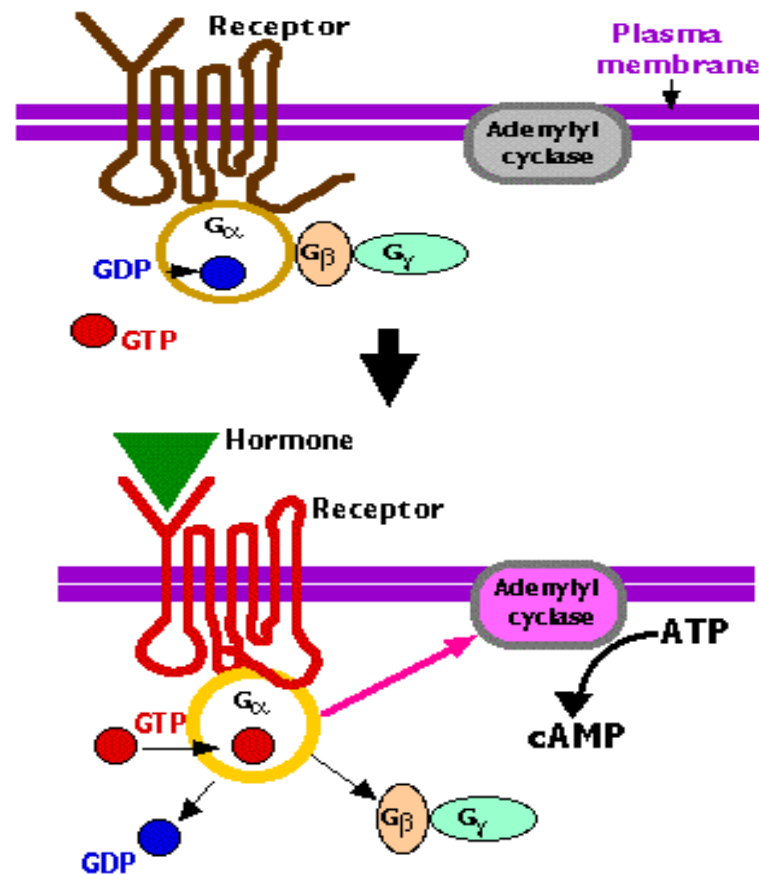


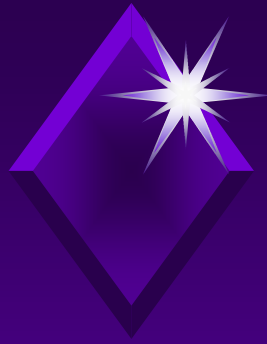
hCG

Pituitary

Placenta

Glycoprotein hormones mechanism of action





Glycoprotein Hormone Subunits

α



hFSH β



hLH β

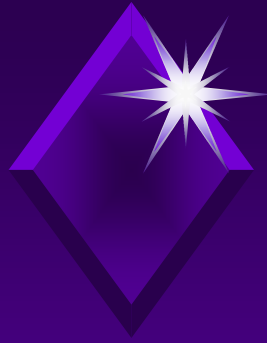


hTSH β



hCG β





CTP

O

|

SerSerSerSerLysAlaProProProSerLeuProSerProSerArgLeu

O

|

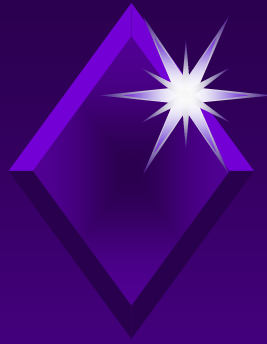
O

|

O

|

Pro GlyProSerAspThrProIleLeuProGln



Glycoprotein Hormone Subunits

α



hCG β



Stop Codon

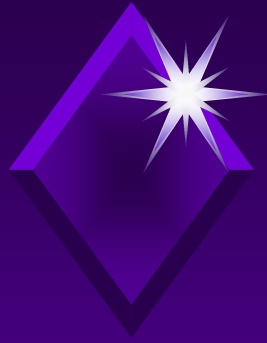
Mutated



hCG β

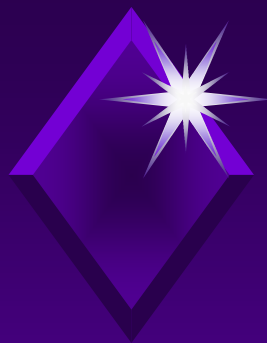


TAA
TAG
TGA



Deletion of CTP from hCG

- No effect on the assembly of subunits
- No effect on receptor binding
- No effect on *in vitro* bioactivity
- Significantly decreased the bioactivity
in vivo



Designing New Analogs

◆ Follicle-Stimulating Hormone

- Used for stimulation of follicular development in women
- Injected daily



Designing New FSH Analog

hFSH Gene

hCG Gene

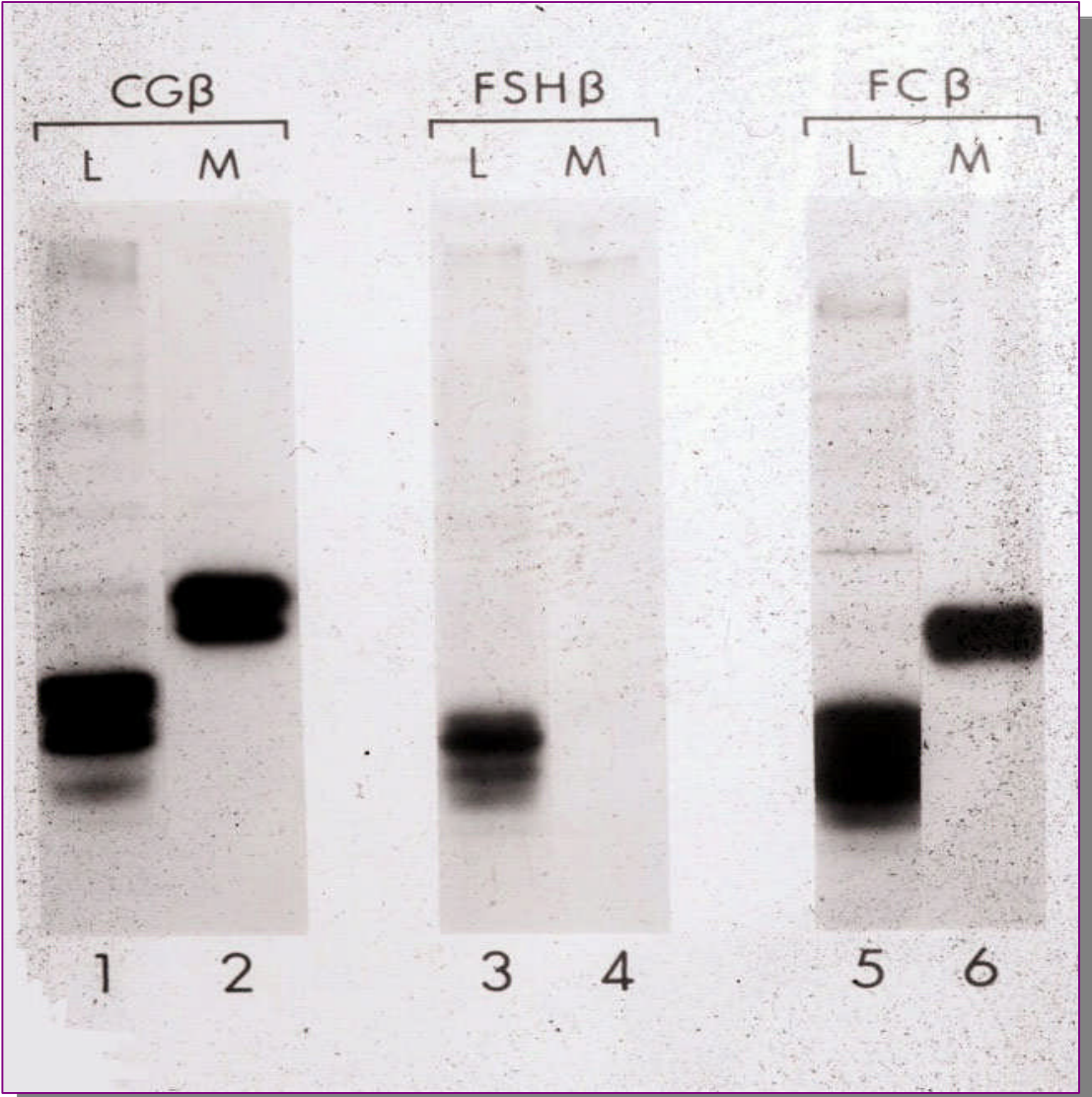
CTP



hFSH Gene

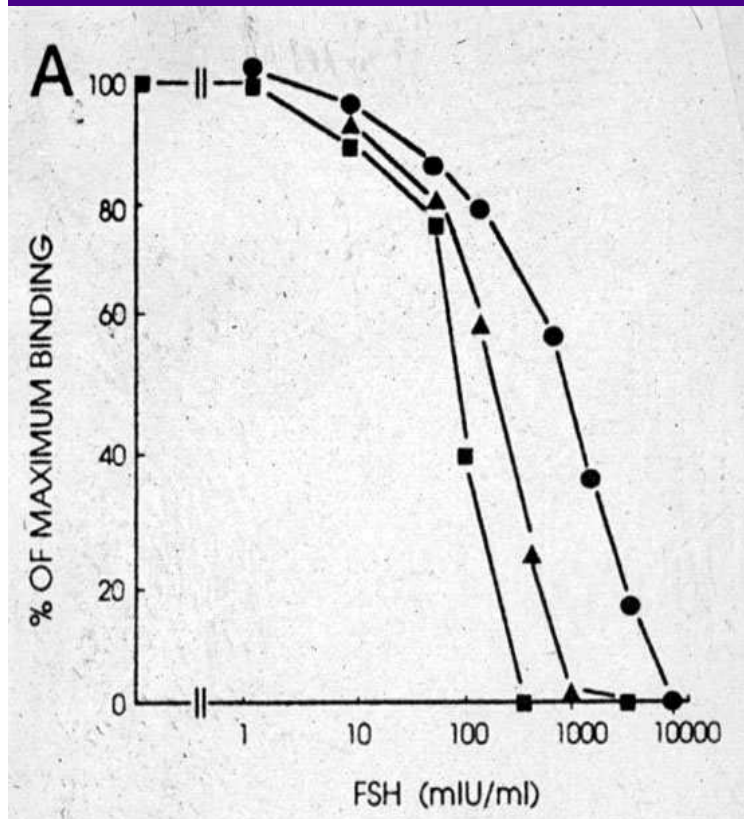
CTP

Secretions

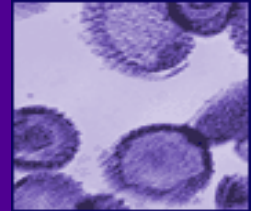
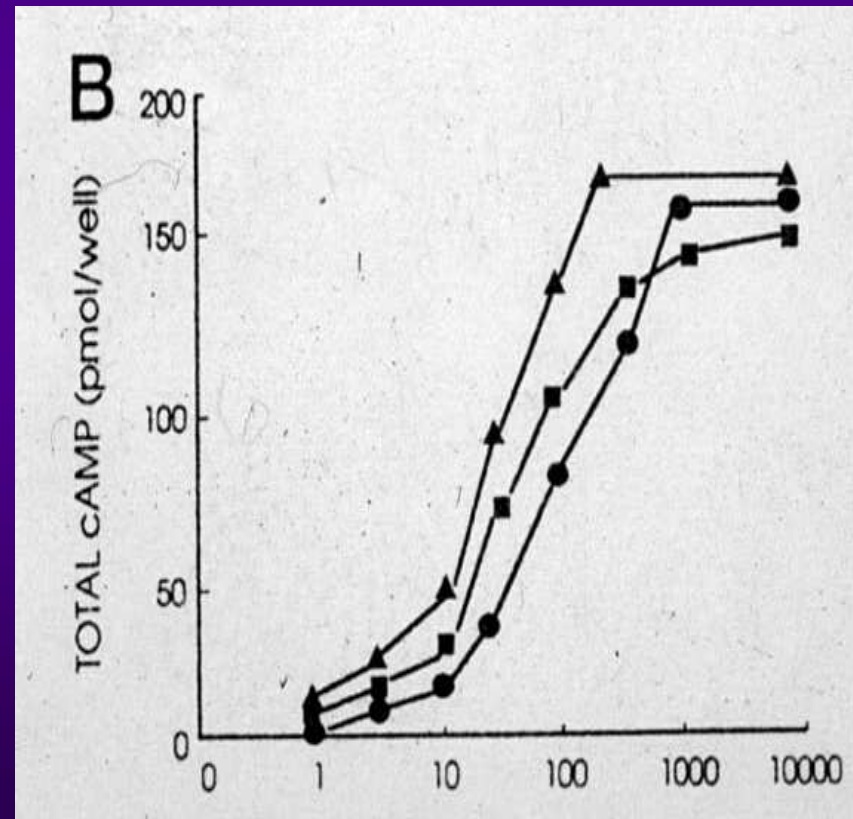


Biological Activity *in vitro*

Receptor binding



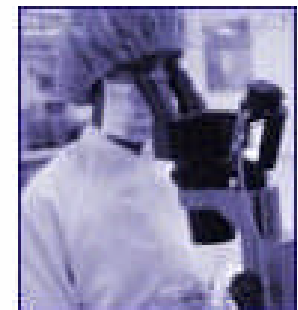
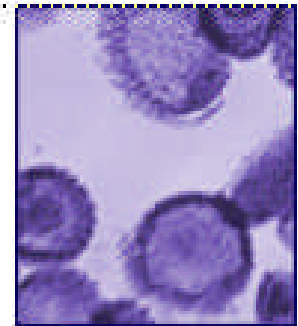
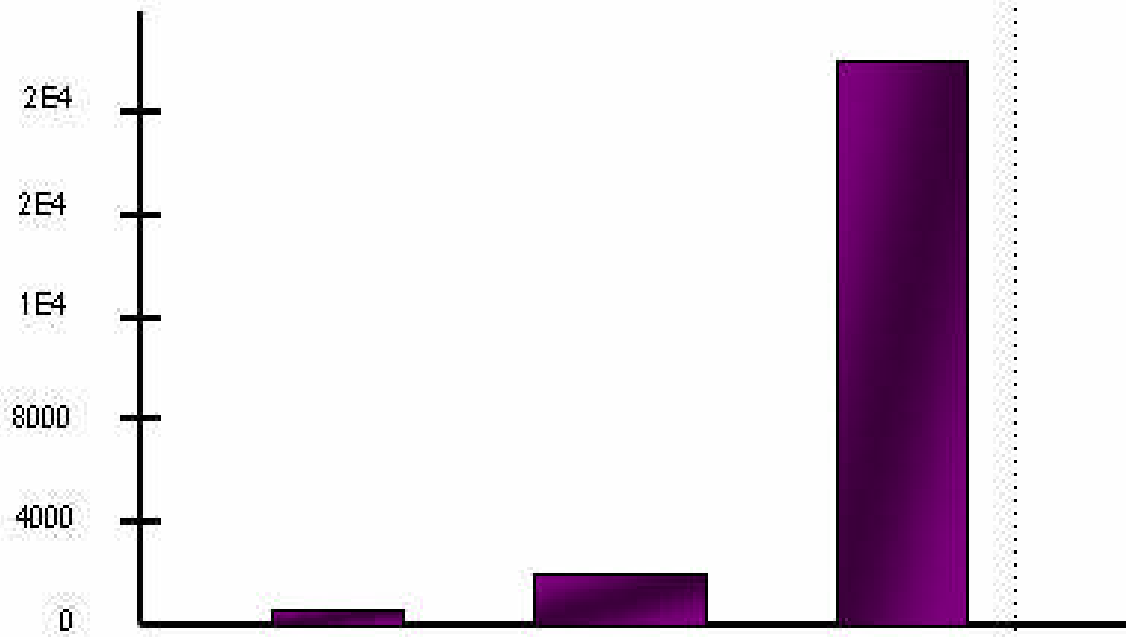
Biological Activity



Biological Activity, *in vivo*

Estrogen (pg/ml)

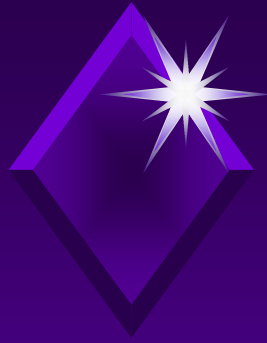
10 IU / 24H x 48H (ip)



Control

FSH-WT

FSH-CTP



CTP

O

|

SerSerSerSerLysAlaProProProSerLeuProSerProSerArgLeu

O

|

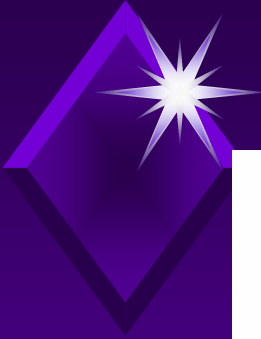
O

|

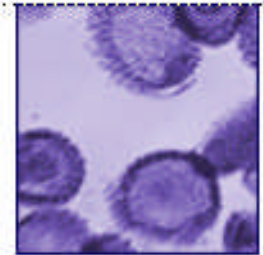
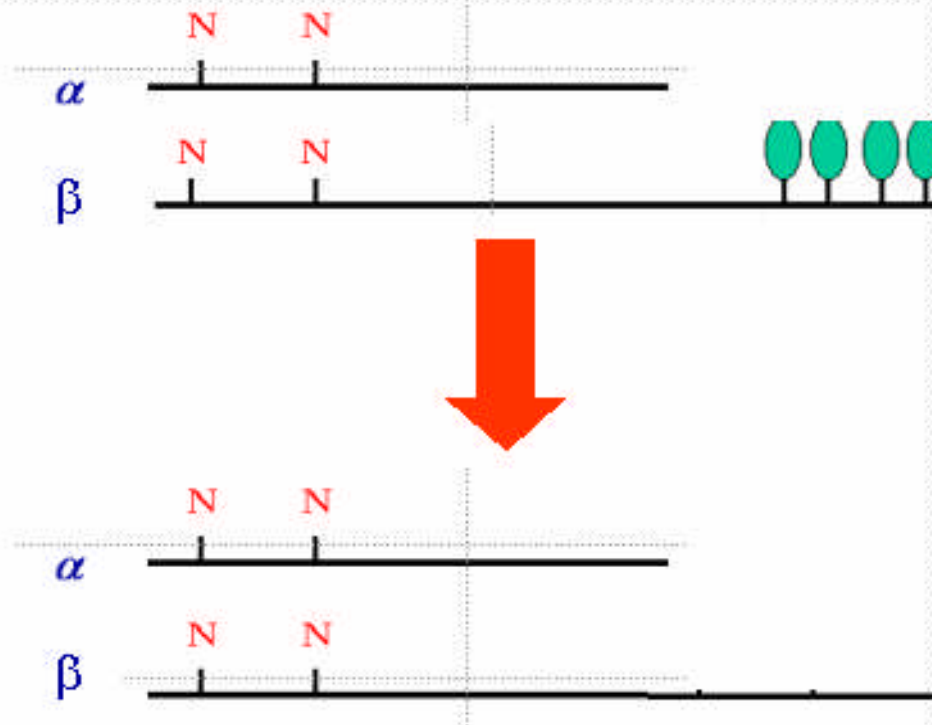
O

|

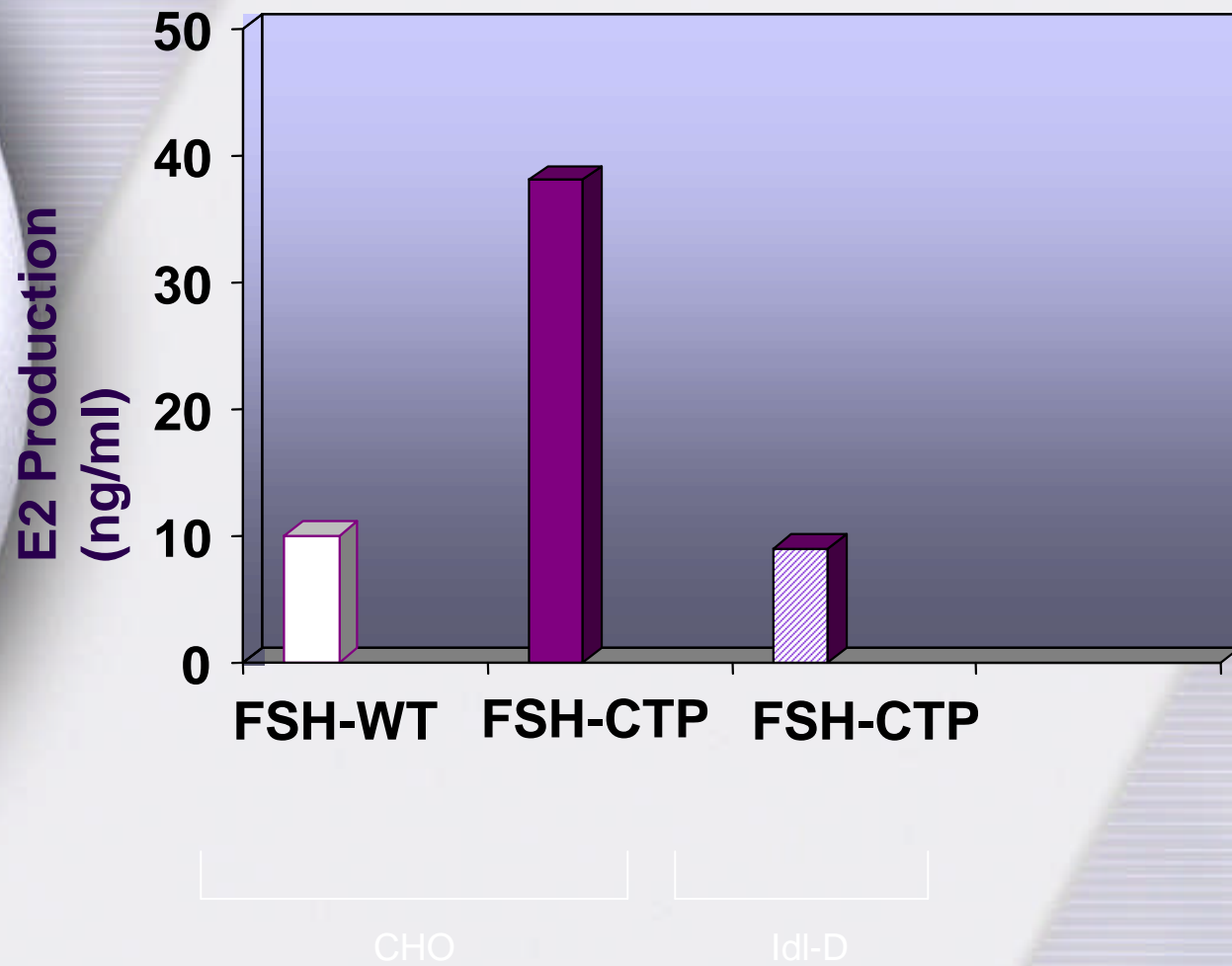
Pro GlyProSerAspThrProIleLeuProGln



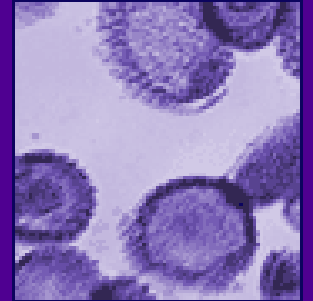
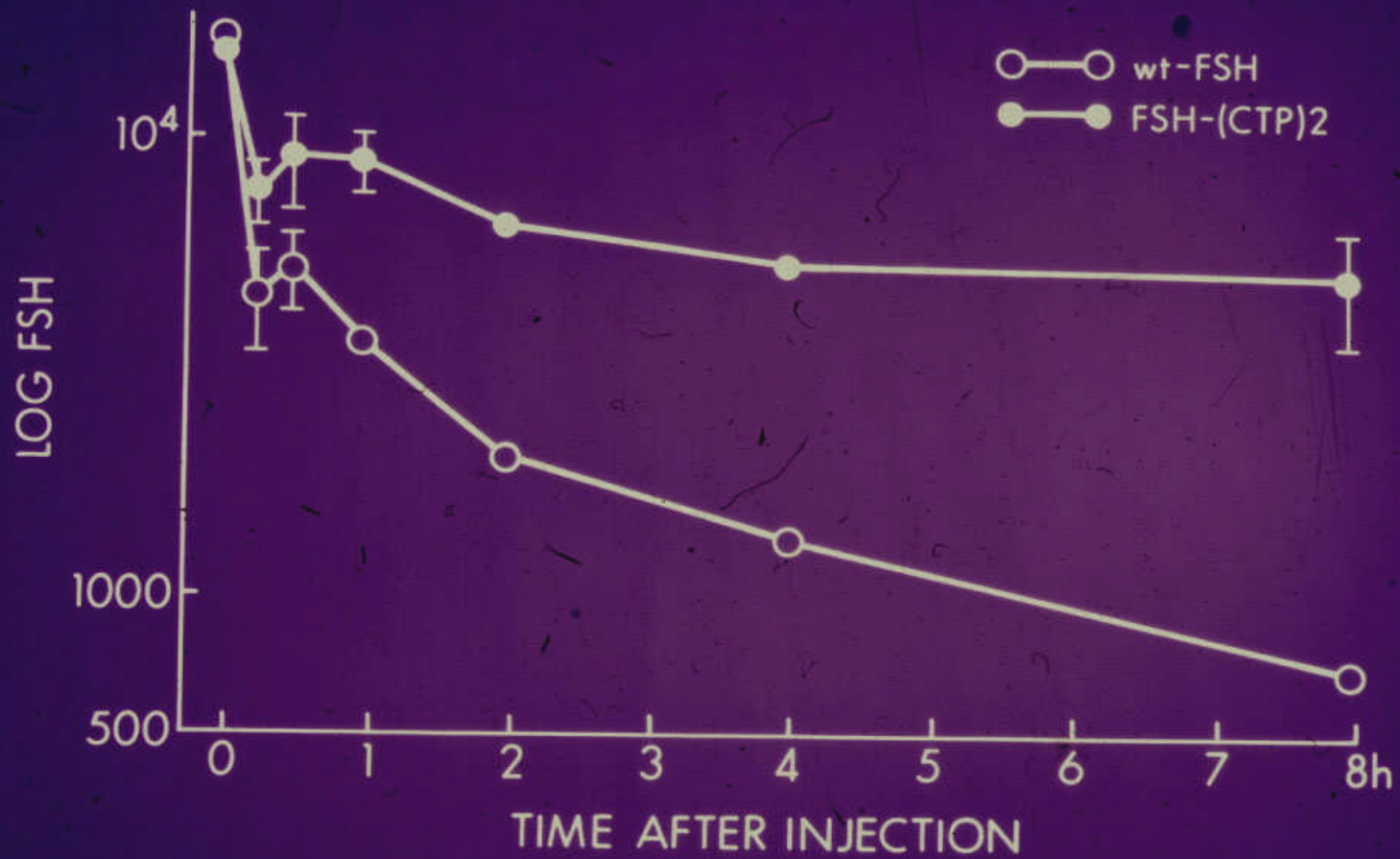
The role of O-linked Oligosaccharides



Biological Activity



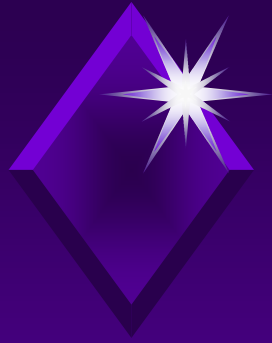
Half Life, in vivo





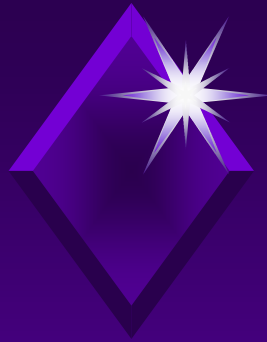
Designing Long Acting Erythropoietin (EPO)

- ◆ **EPO is Produced in the kidney**
- ◆ **Glycoprotein hormone involved with growth and development of mature red blood cells from erythrocyte precursor cells.**



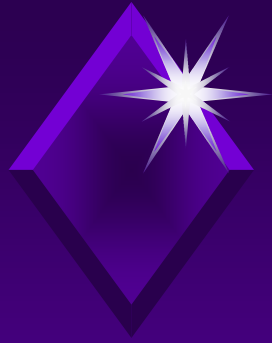
Therapeutic use of EPO

- **EPO has a wide clinical use;**
 - Several forms of *anemia*, including those associated with renal failure, HIV infection, blood loss, and chronic disease.
 - EPO injected three times/week



EPO Market

- ◆ **Treatment for chronic dialysis patients estimated between \$8,000 and \$10,000 per patient per year**
- ◆ **Market volume of its usage worldwide estimated at around \$6 billion per year**



Designing New EPO Analog

hEPO Gene

hCG β Gene

CTP

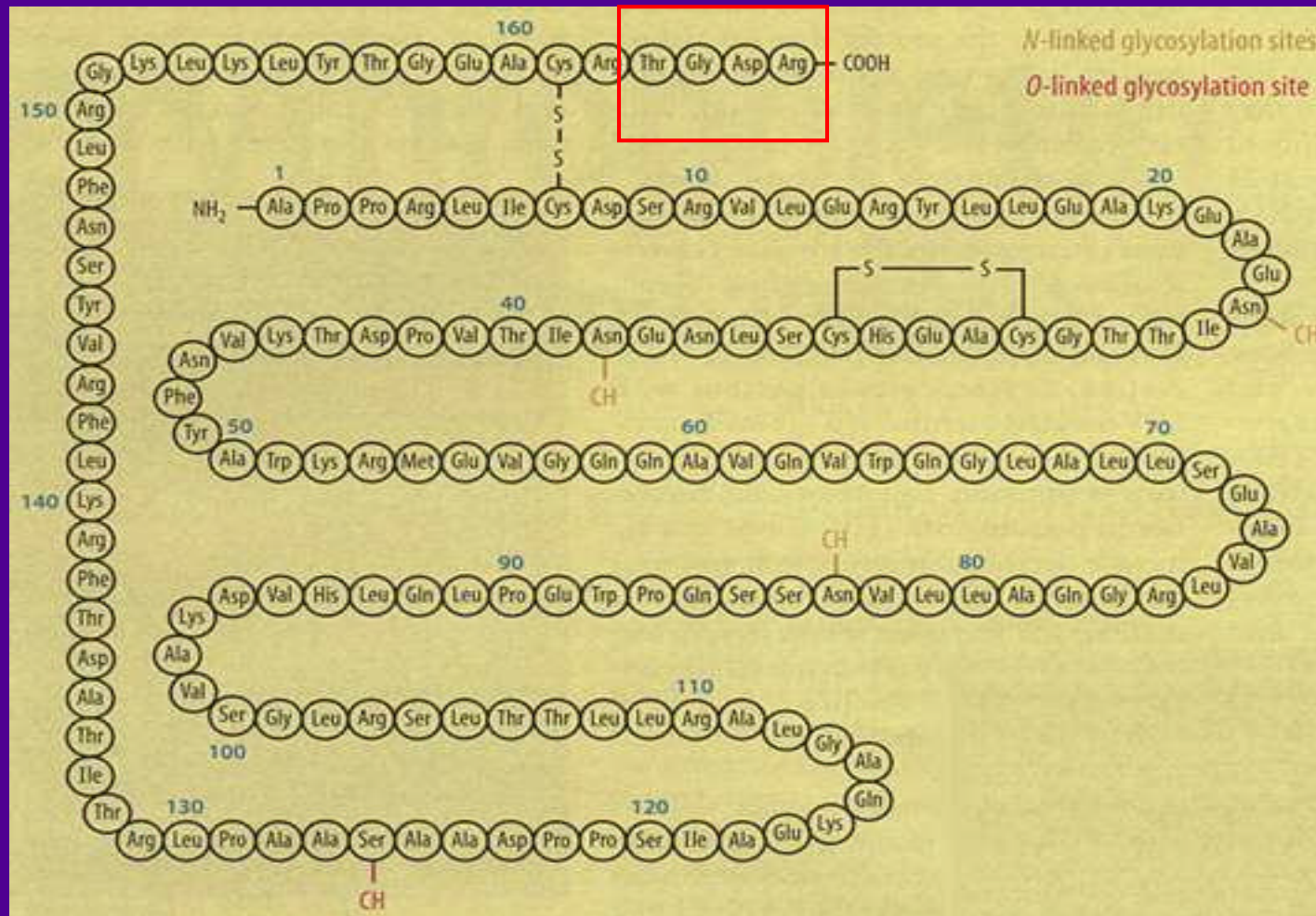


hEPO Gene

CTP

Human EPO

Amino Acid Sequence



EPO-CTP Construction

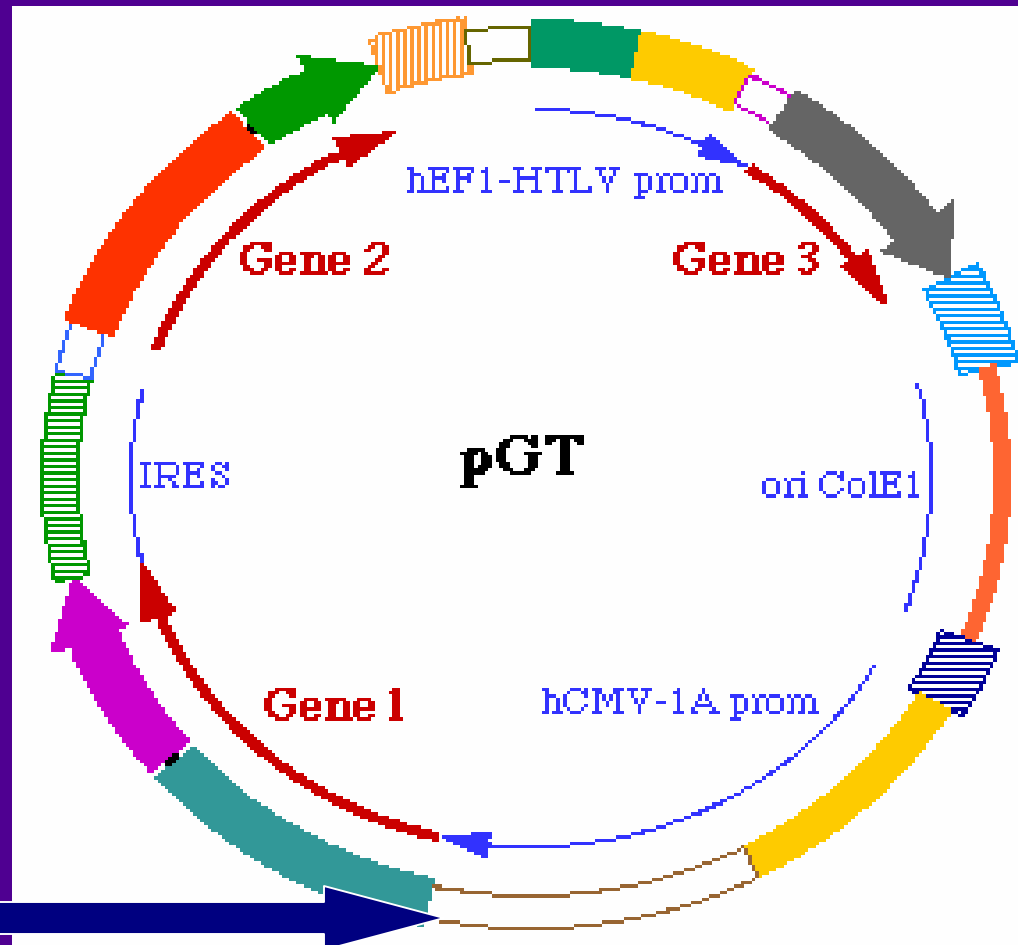


| | | | | | | | |
|---|----|----|-----|---|---|---|---|
| N | N | N | O | O | O | O | O |
| | | | | | | | |
| NH ₂ ...Asn-X-Thr...Asn-X-Thr...Asn-X-Ser.....Ser.....Ser...Ser...Ser...Ser.....COOH | | | | | | | |
| 24 | 38 | 83 | 126 | | | | |

Human EPO-CTP cDNA Sequence

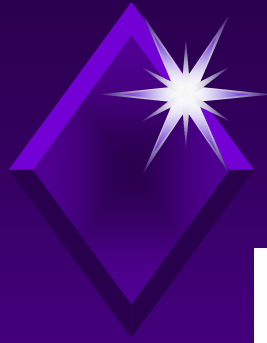
ACCAGATCTACCGGTCATCATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCT
CCTGTCCTTCTGTCGCTCCCTCTGGGCCTCCAGTCCTGGGCGCCCCACCACGCCT
CATCTGTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAGAA
TATCACGACGGGCTGTGCTGAACACTGCAGCTTGAATGAGAATATCACTGTCCCAGA
CATCAAAGTTAATTTCTATGCCTGGAAGAGGATGGAGGTCGGGCAGCAGGCCGTAGA
AGTCTGGCAGGGCCTGGCCCTGCTGTCCGAAGCTGTCCTGCGGGGCCAGGCCCTGTT
GGTCAACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTCAG
TGGCCTTCGCAGCCTCACC ACTCTGCTTCGGGCTCTGGGAGCCAGAAGGAAGCCAT
CTCCCCCTCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTT
CCGCAA ACTCTTCCGAGTCTACTCCAATTTCTCCGGGGAAAGCTGAAGCTGTACAC
AGGGGAGGCCTGCAGGACAGGGGACAGATCCTCTTCCTCAAAGGCCCTCCCCGAG
CCTTCCAAGTCCATCCCGACTCCCGGGGCCCTCGGACACCCCGATCCTCCCACAATA
AAGGCTTCTGGATCCGCACTCTGGAGGTAA

Cloning Plasmid

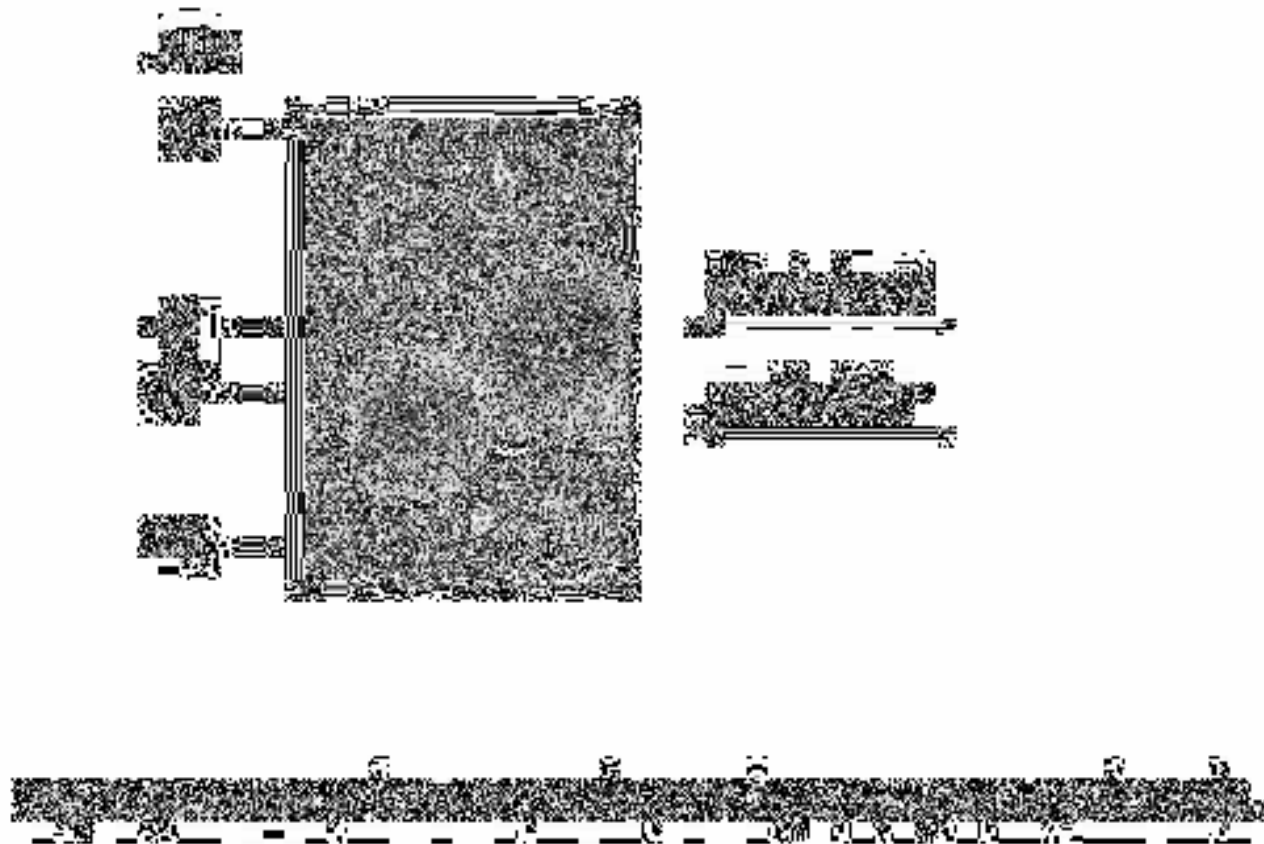


Cloning site

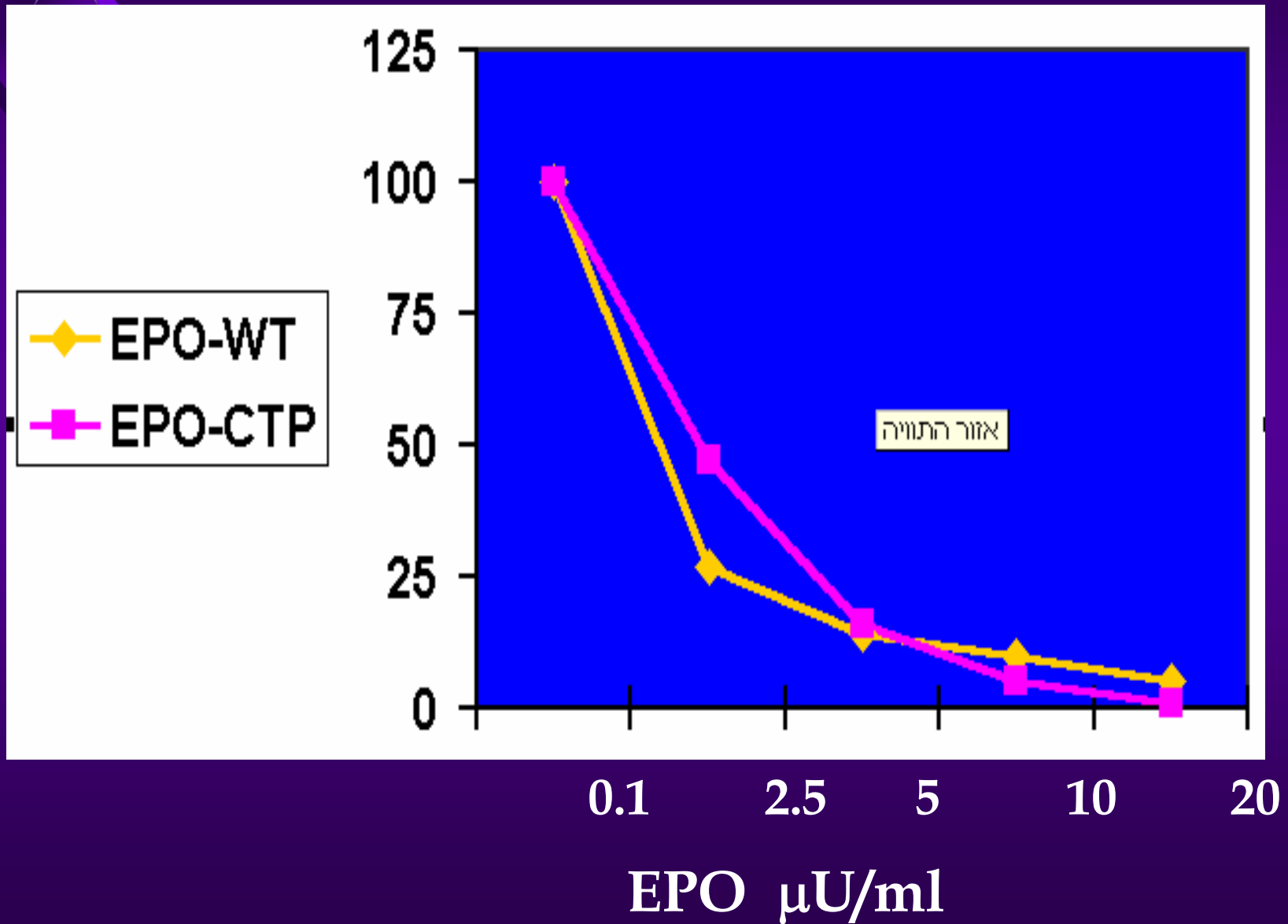
invivogen



Secretion



Receptor Binding



In vitro Bioactivity

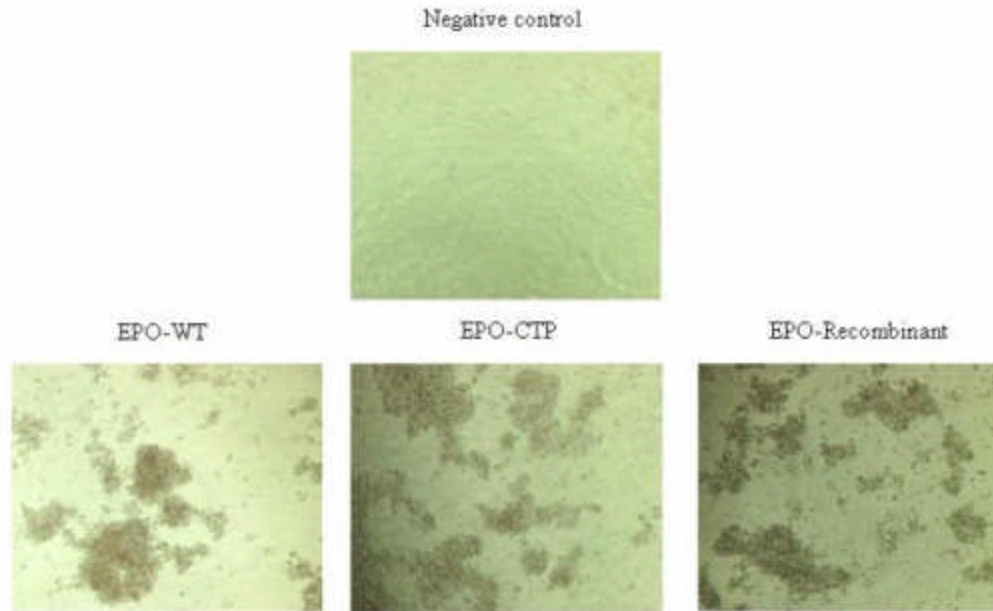
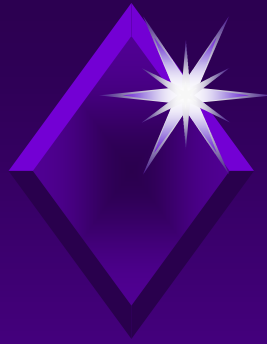
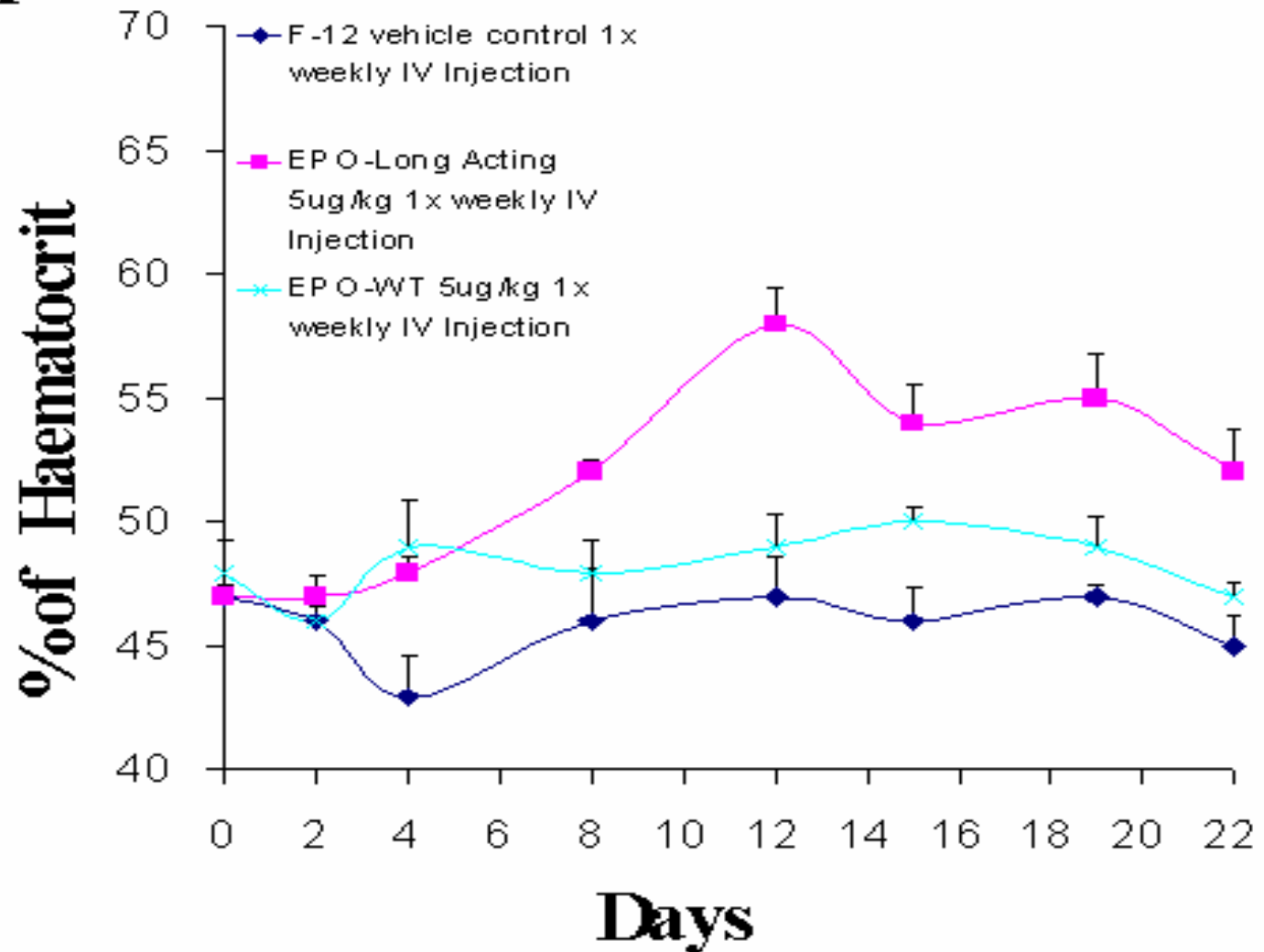


Fig. 6. BFU-E colonies stimulated by EPO analogs. Purified cells from human peripheral blood were incubated for 20 days. BFU-E colonies were identified by microscopic examination and photographed.

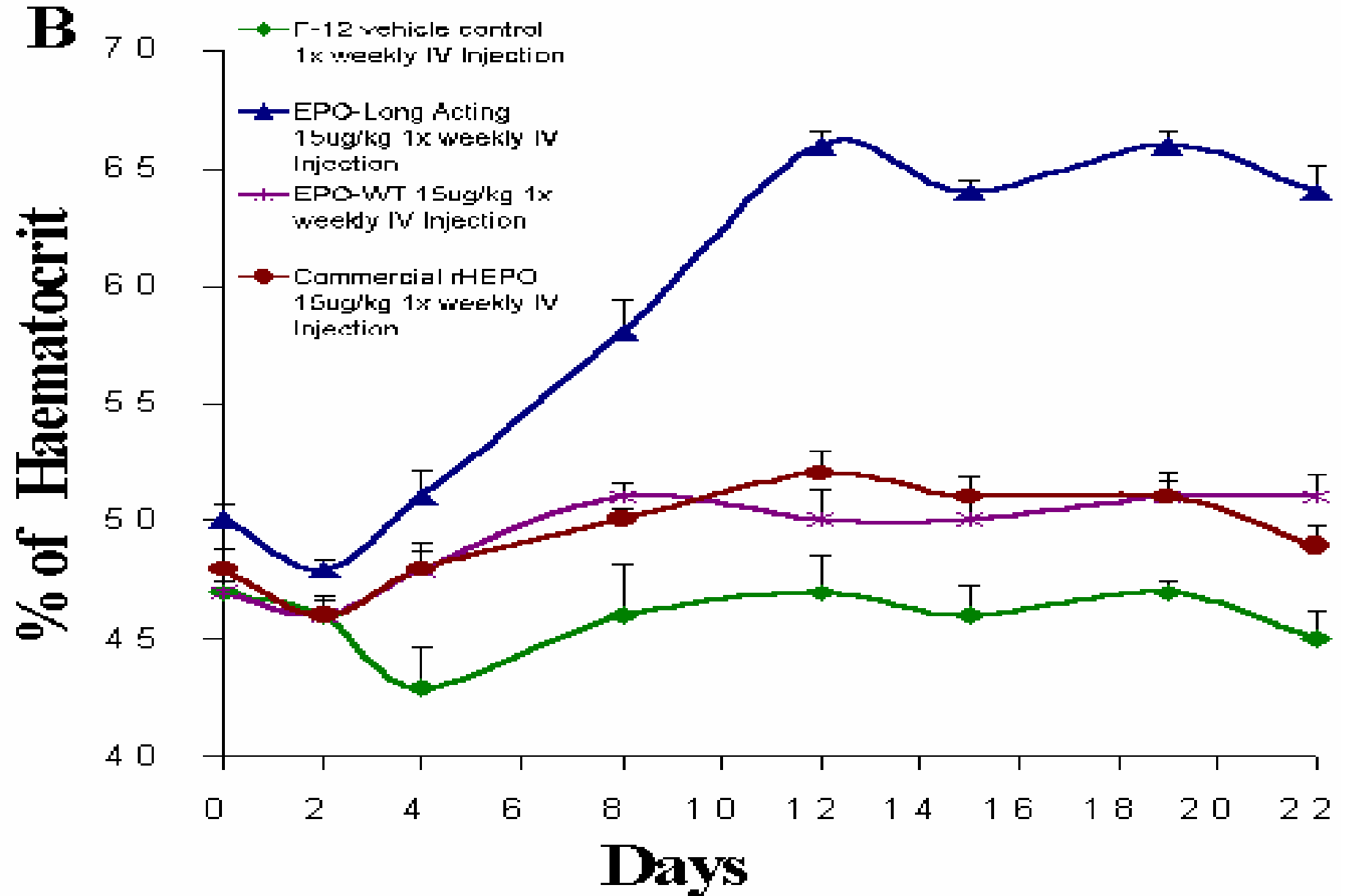


In vivo Bioactivity

A

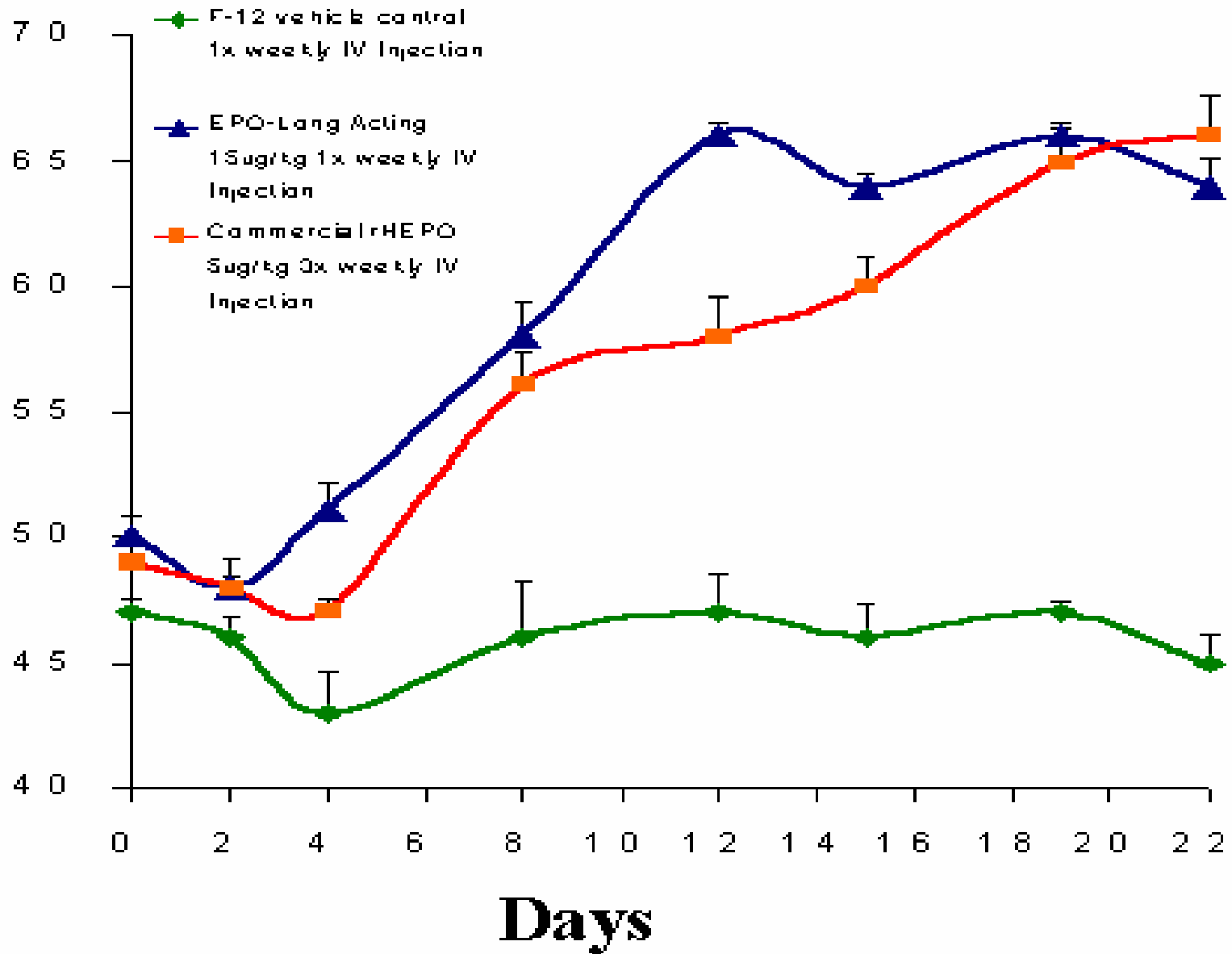


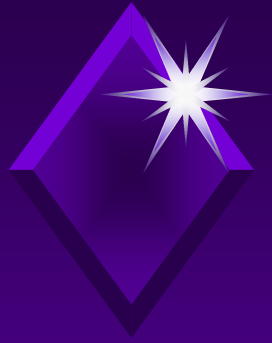
In vivo Bioactivity



In vivo Bioactivity

C
% of Haematocrit

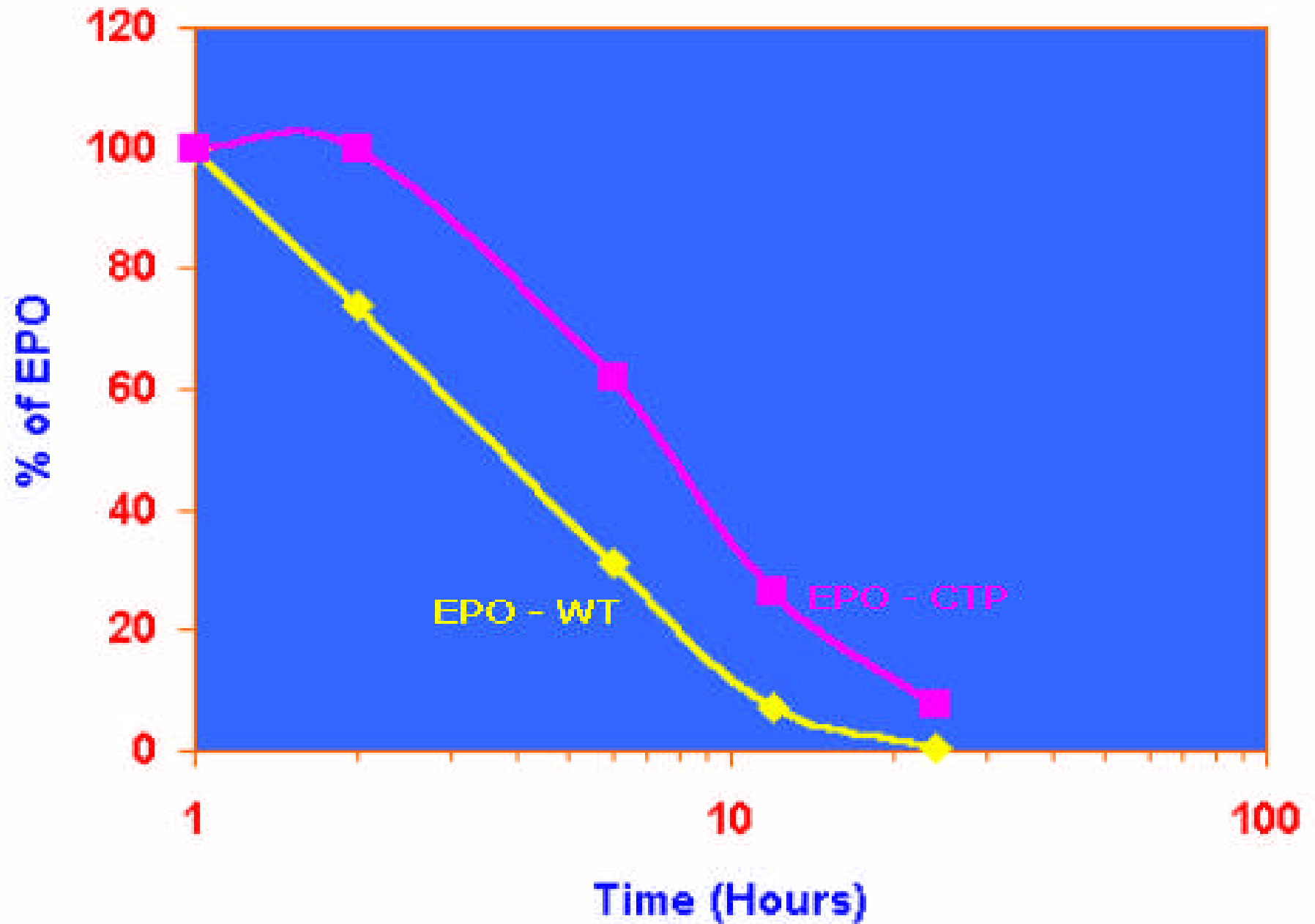


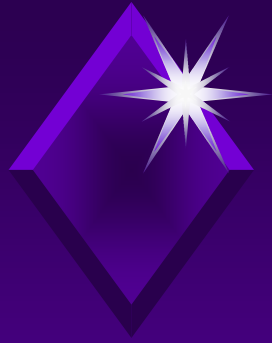


EPO – long acting

- ◆ **Bind with high affinity to hEPO receptor**
- ◆ **The half – life is 2-3 times higher than EPO-WT**

In vivo half-life





Summary

- ◆ *O*- linked oligosaccharides have no role in receptor binding and *in vitro* bioactivity of the hormone
- ◆ Ligation of the CTP to different proteins, resulted in increasing of the *in vivo* half-life and bioactivity.



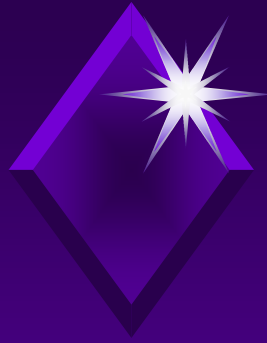
Structure-Function of hTSH

α Subunit



hTSH β Subunit





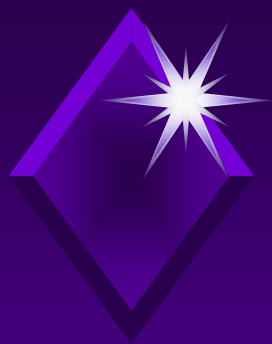
Carbohydrate Recognition Signal

- ◆ *N*-linked Oligosacharides

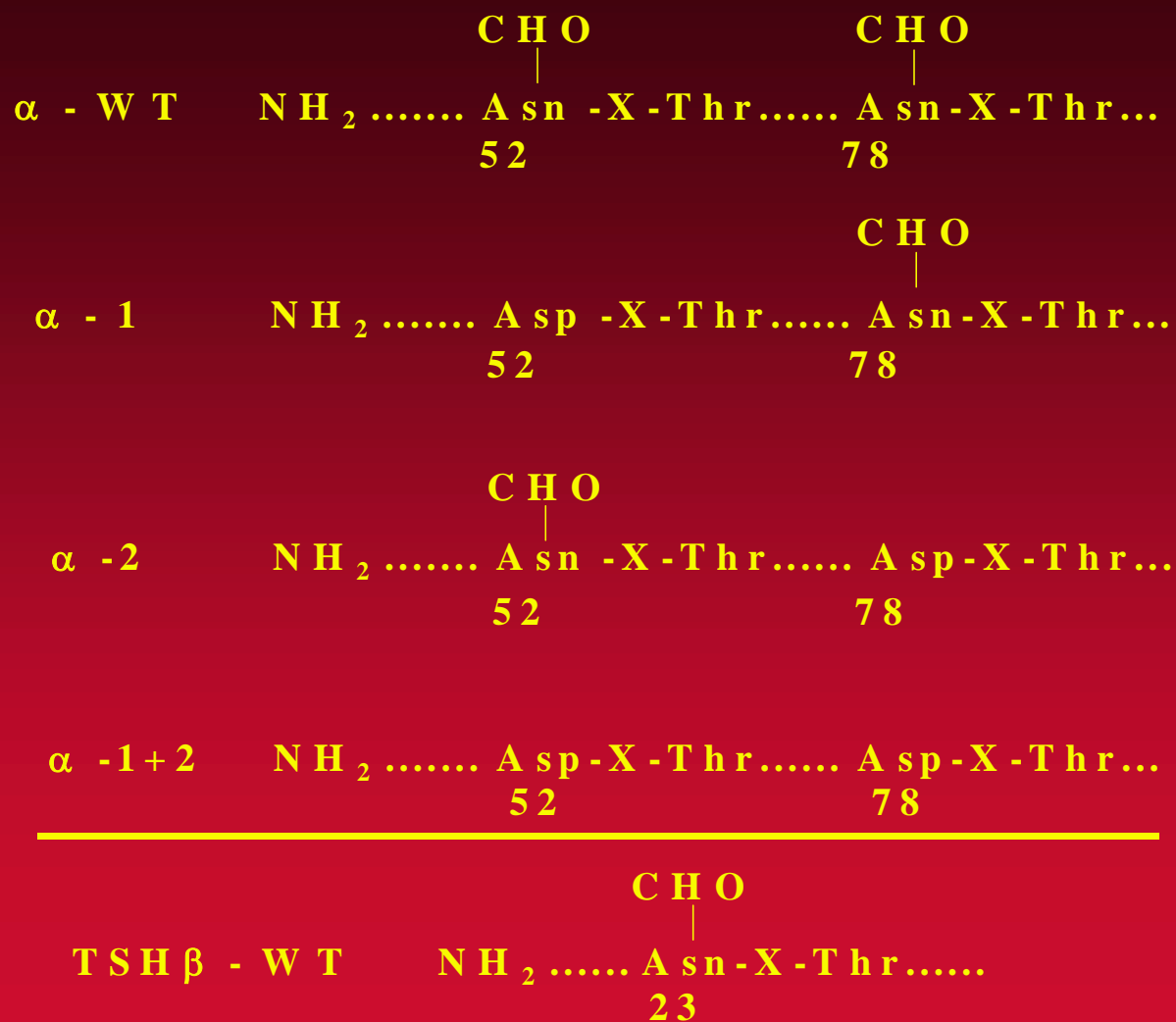
Asn – X – Ser/Thr

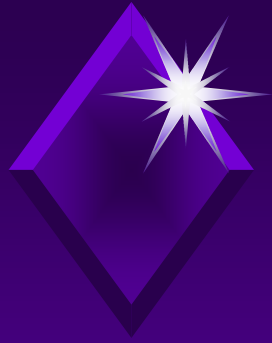
- ◆ *O*-linked oligosacharides

Ser/Thr



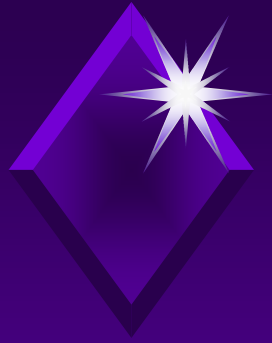
hTSH Variants





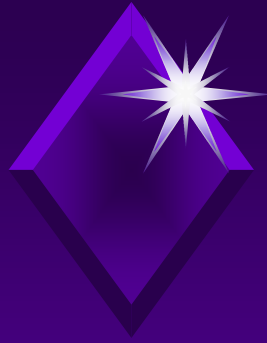
hTSH Mutants

- ◆ **Expressed in CHO Cells**
- ◆ **Bioactivity was detected by measuring the ability of the hTSH to stimulate cAMP formation and T3 secretion from cultured human thyroid follicles.**

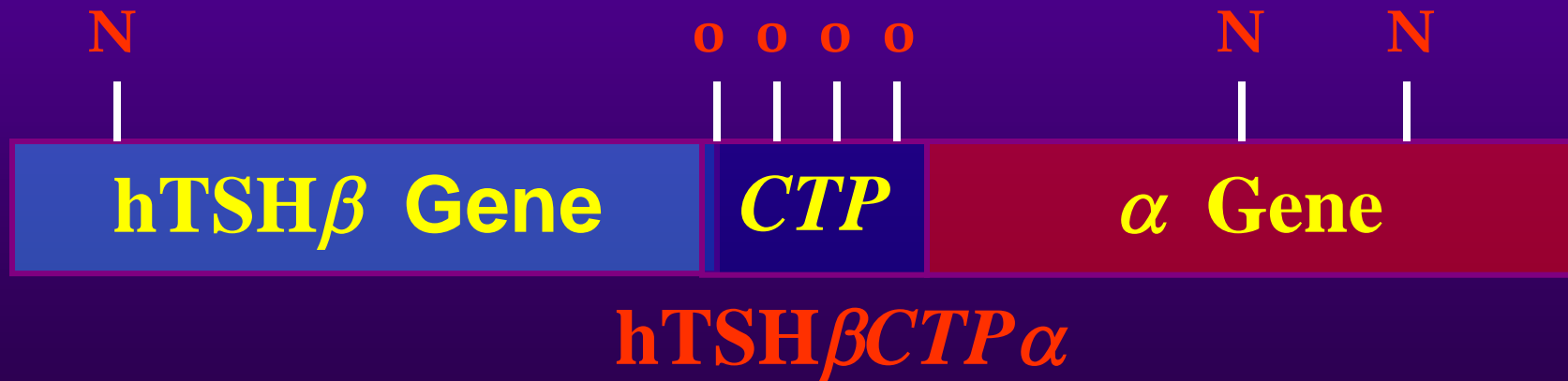


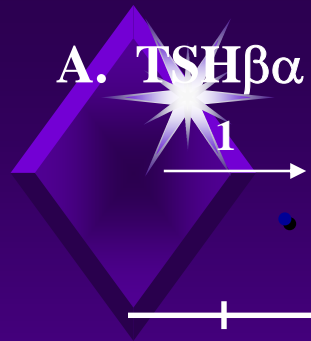
hTSH Mutants

- ◆ **Reduction in the protein secretion**
- ◆ **No effect on receptor binding**
- ◆ **Reduction in biological activity**



hTSH Single Chain





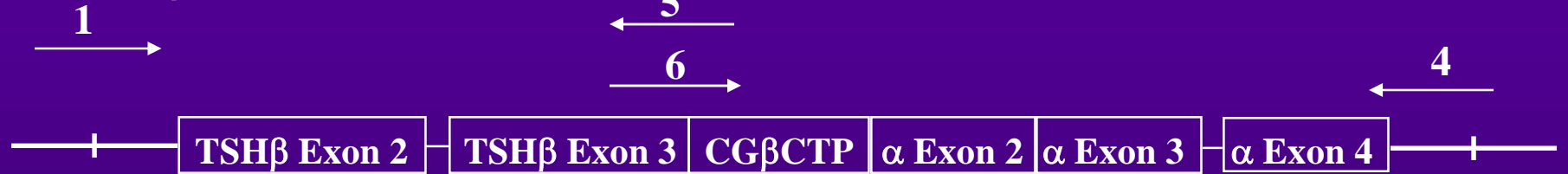
BamHI

Sal I

| | | | | | | |
|-----|-----|-----|--|-----|-----|-----|
| TTT | TCT | GTC | | GCT | CCT | GAT |
| Phe | Ser | Val | | Ala | Pro | Asp |
| 136 | 137 | 138 | | 1 | 2 | 3 |

TSH β Exon 3 ← → α Exon 2

B. TSH β CTP α



BamHI

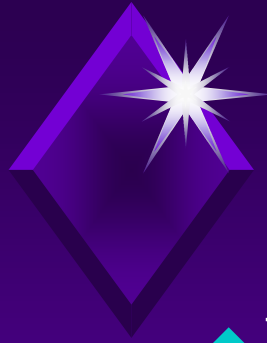
Sal I

| | | | | | | | | | | | | | | |
|-----|-----|-----|--|-----|-----|-----|-----|-----|-----|-----|--|-----|-----|-----|
| TTT | TCT | GTC | | TCC | TCT | TCC | --- | CTC | CCA | CAA | | GCT | CCT | GAT |
| Phe | Ser | Val | | Ser | Ser | Ser | --- | Leu | Pro | Gln | | Ala | Pro | Asp |
| 136 | 137 | 138 | | 118 | 119 | 120 | --- | 143 | 144 | 145 | | 1 | 2 | 3 |

TSH β Exon 3 ← →

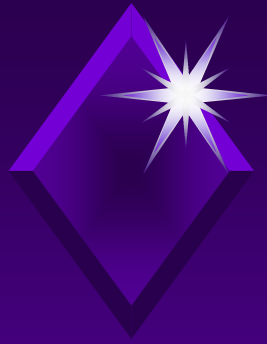
CG β CTP

← → α Exon 2

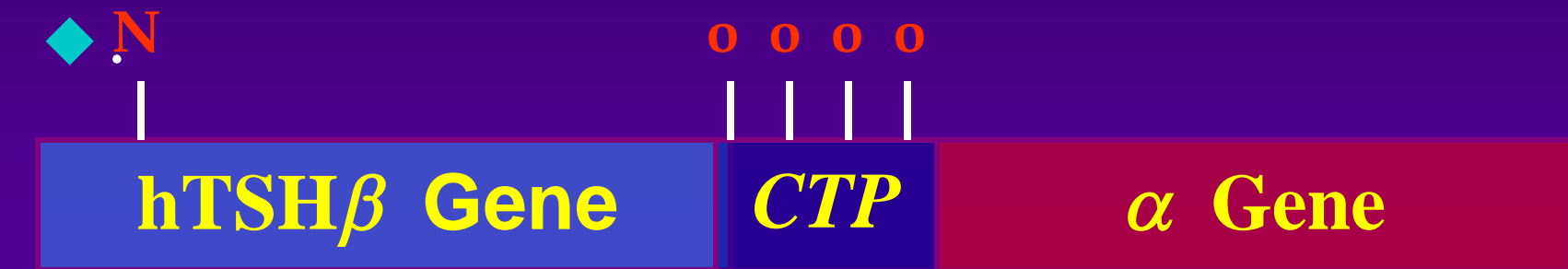


hTSH Single Chain

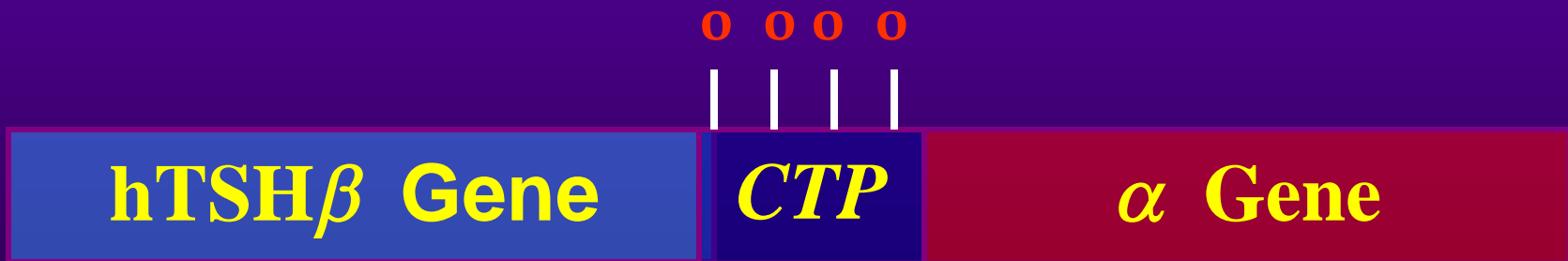
- ◆ **Expressed in CHO cells**
- ◆ **Binds to TSH Receptor in high affinity as well as the TSH-WT**
- ◆ **Biologically active**



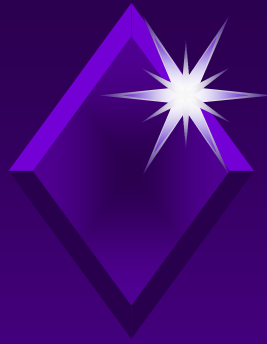
hTSH – Single Chain Variants



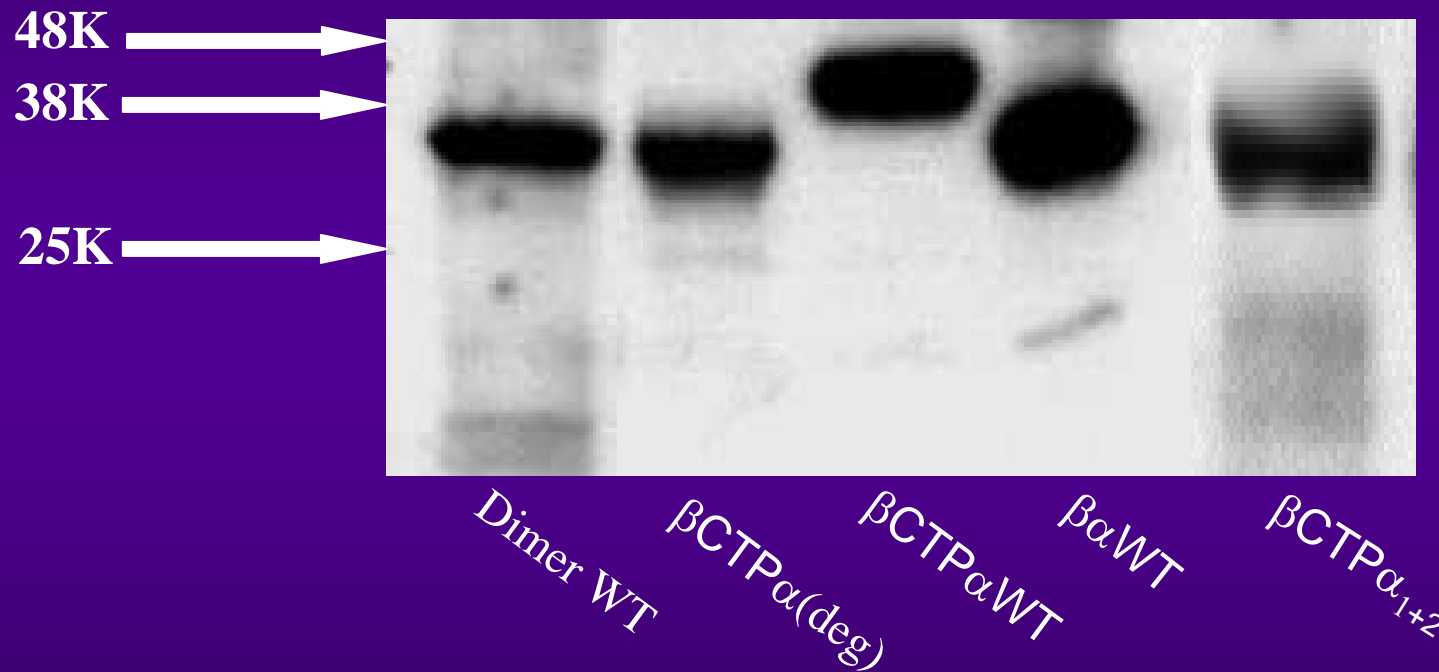
hTSH β CTP α 1+2



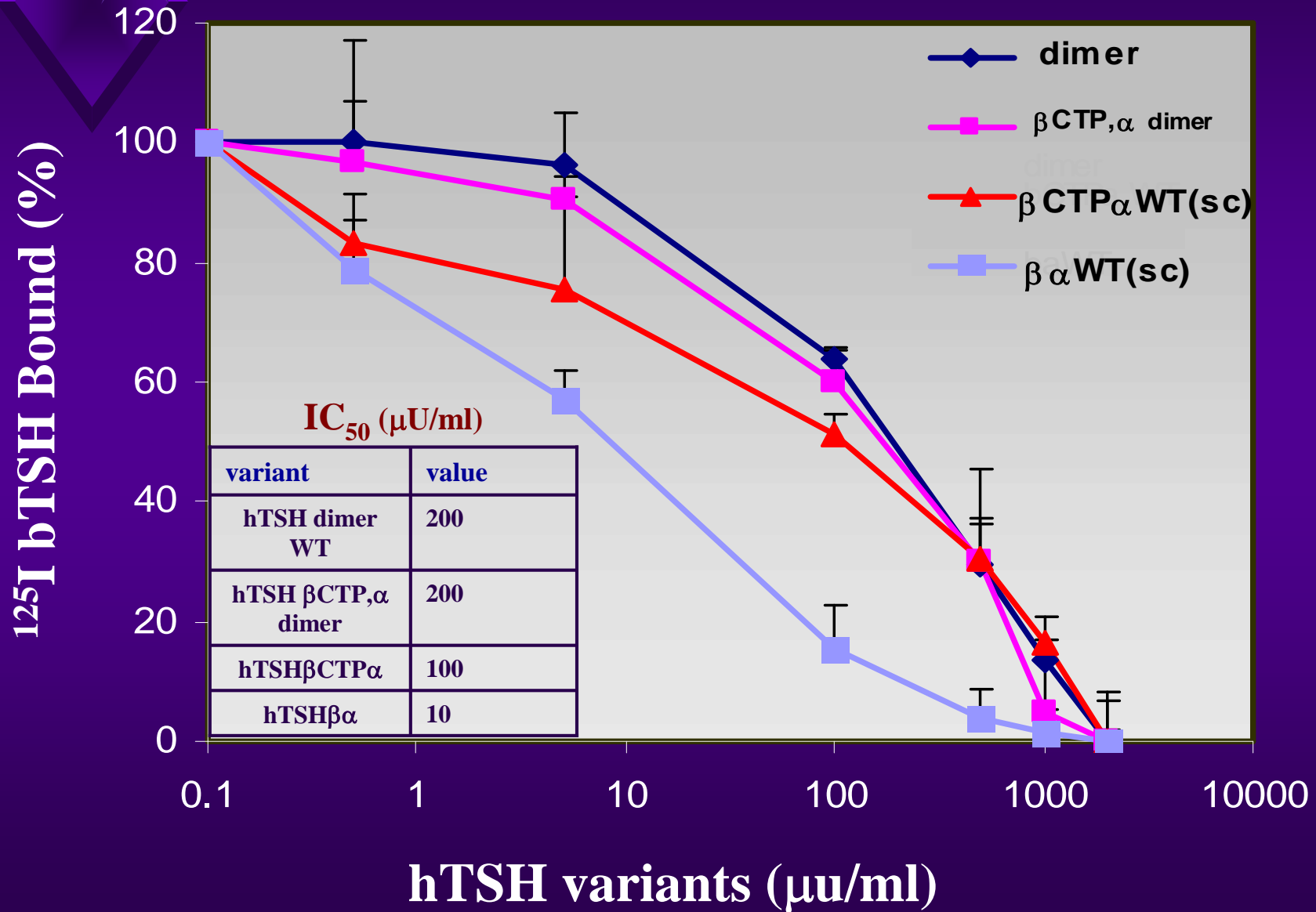
hTSH β CTP α deg



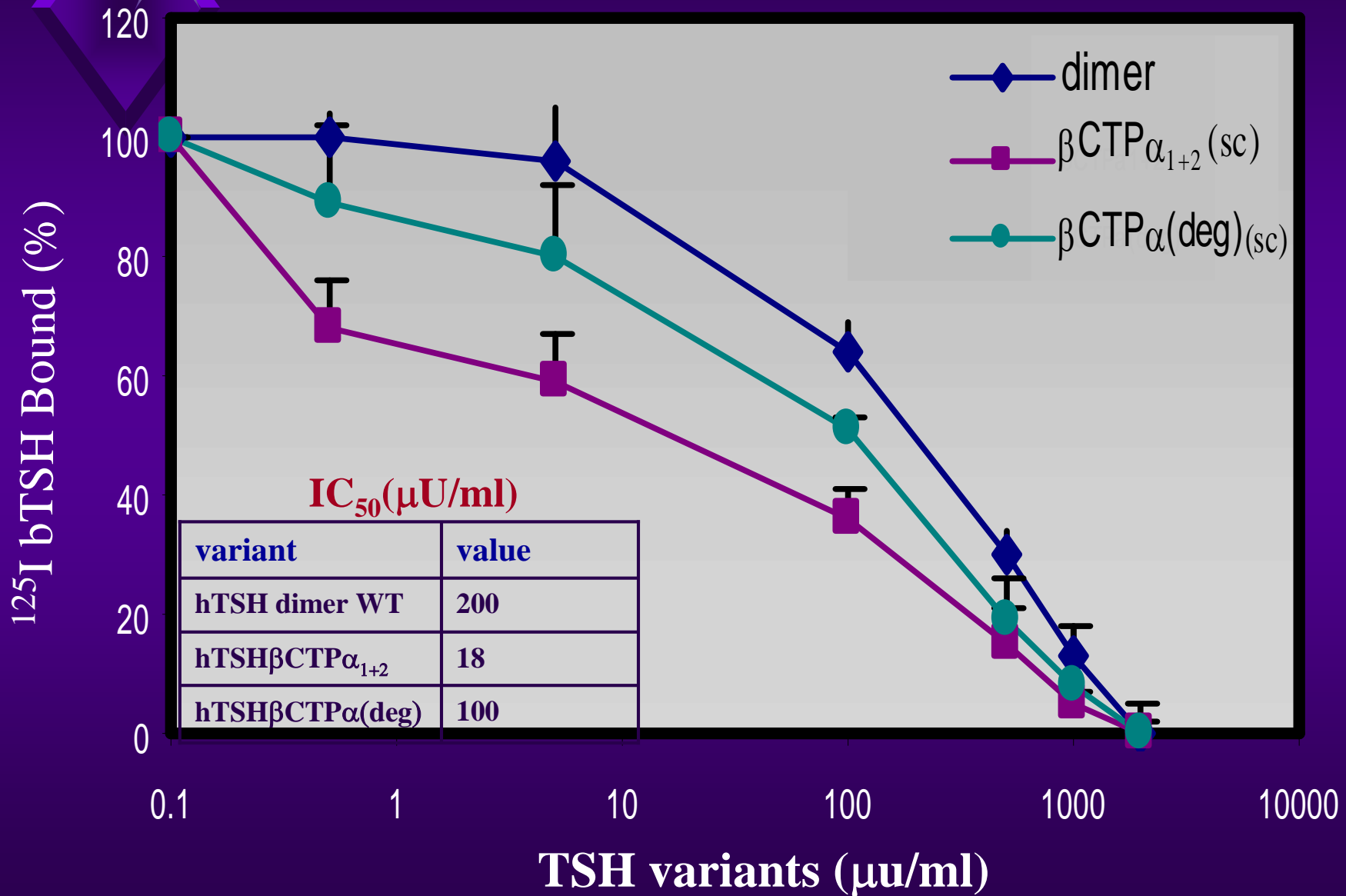
Secretion of TSH variants



Receptor Binding TSH (WT)

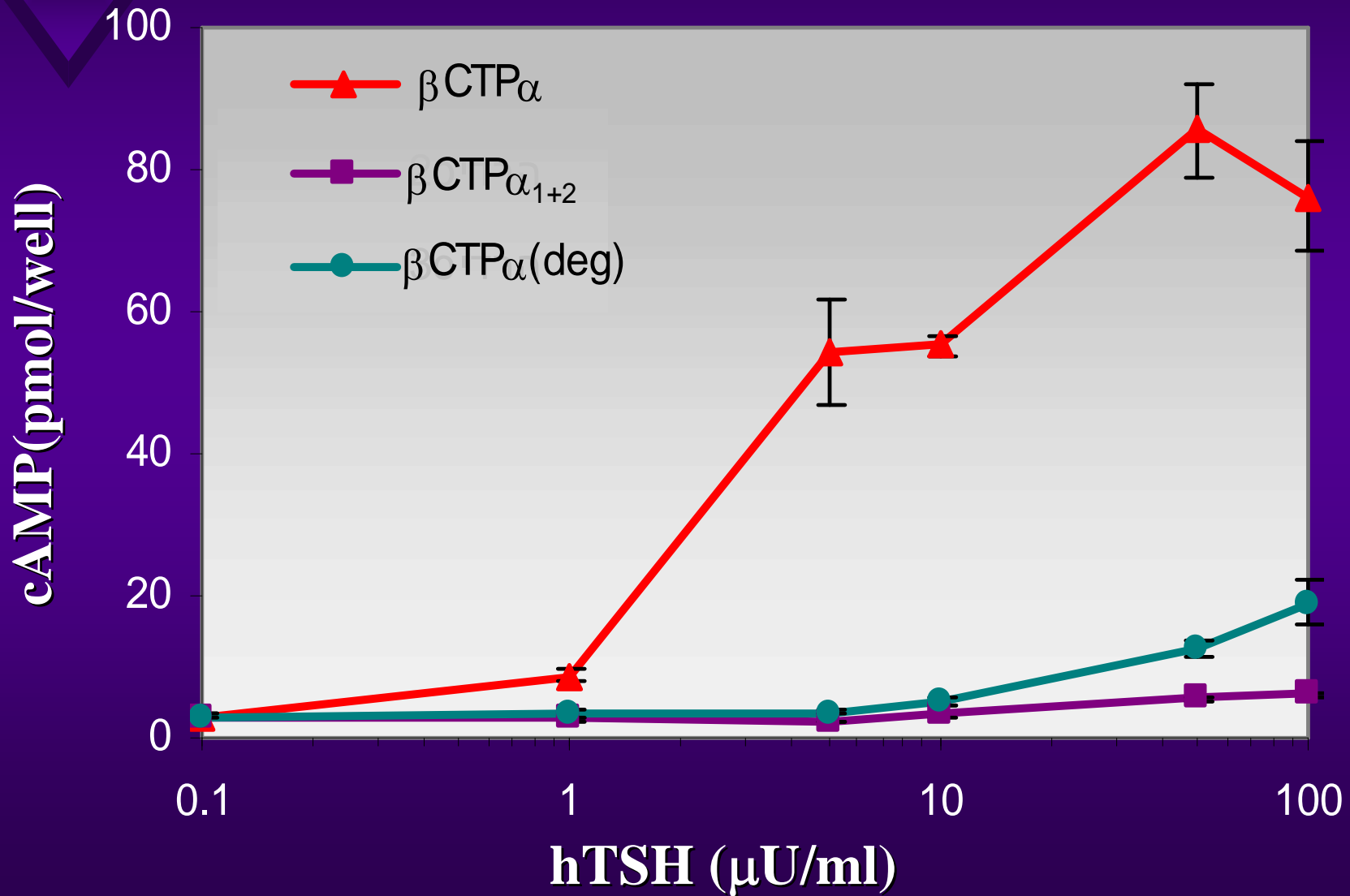


Receptor Binding TSH (Mutants)



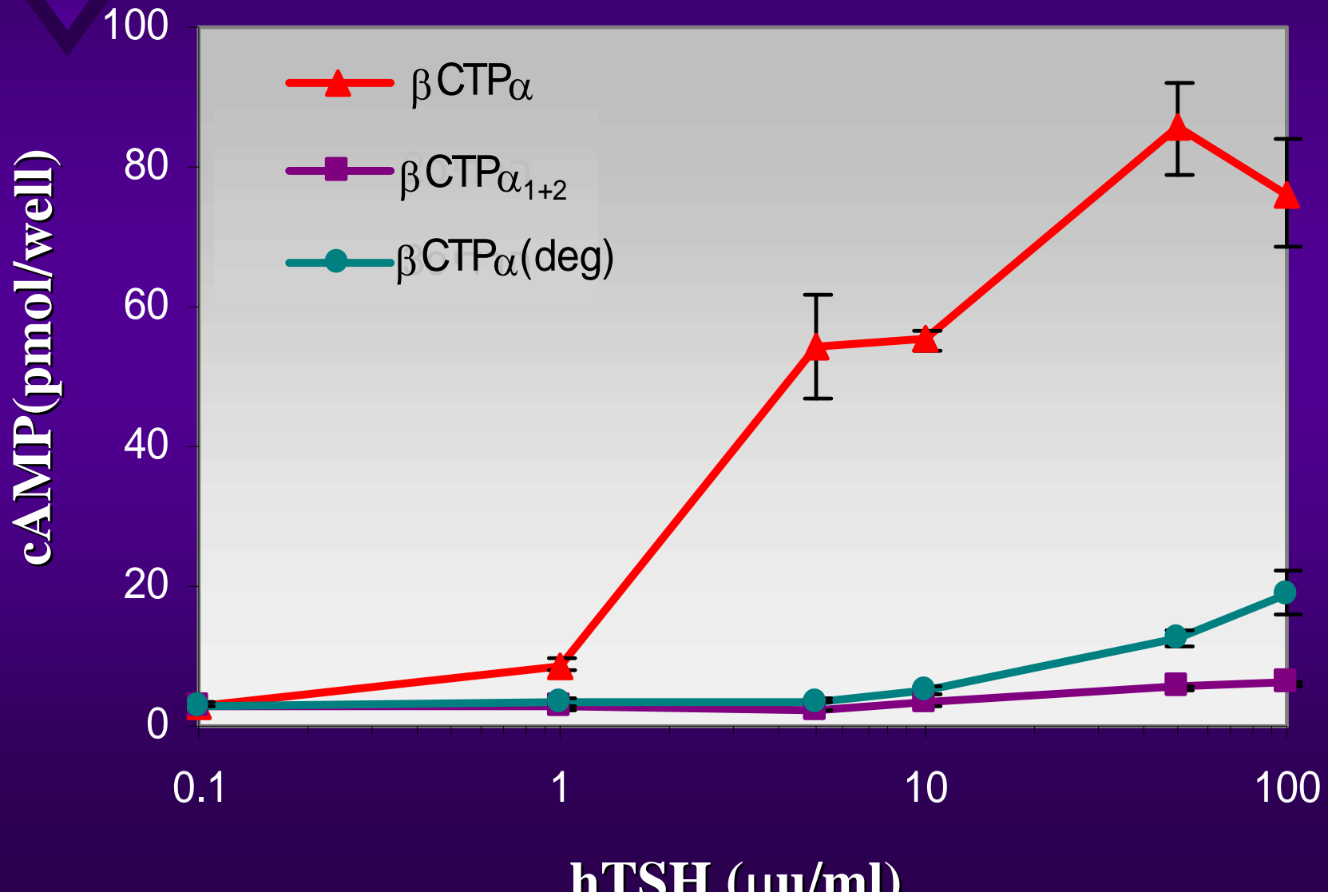


In vitro Bioactivity

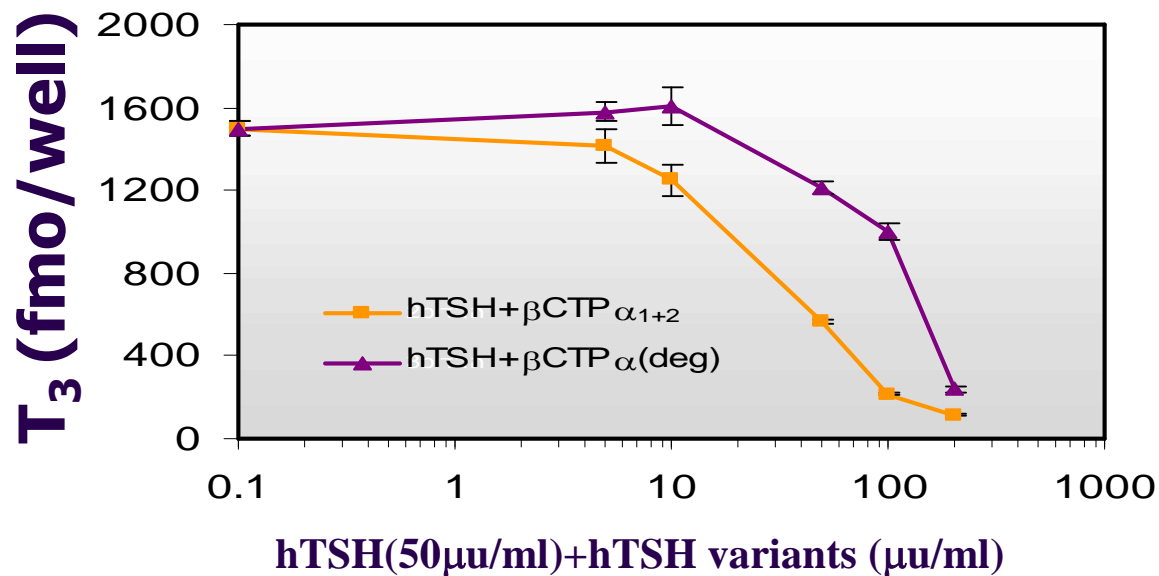
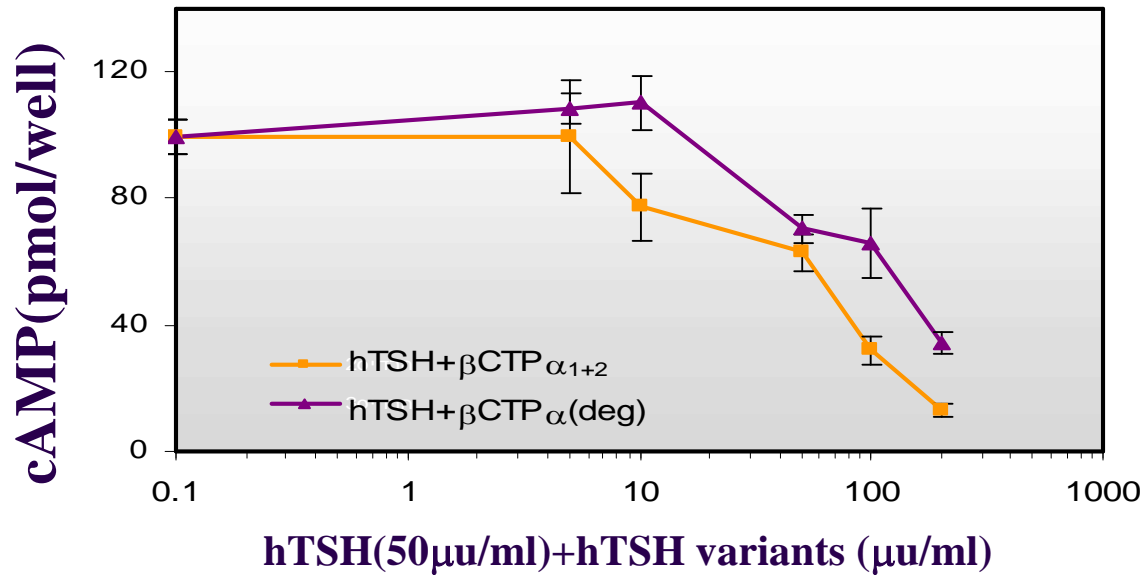




In vitro Bioactivity



TSH Antagonist

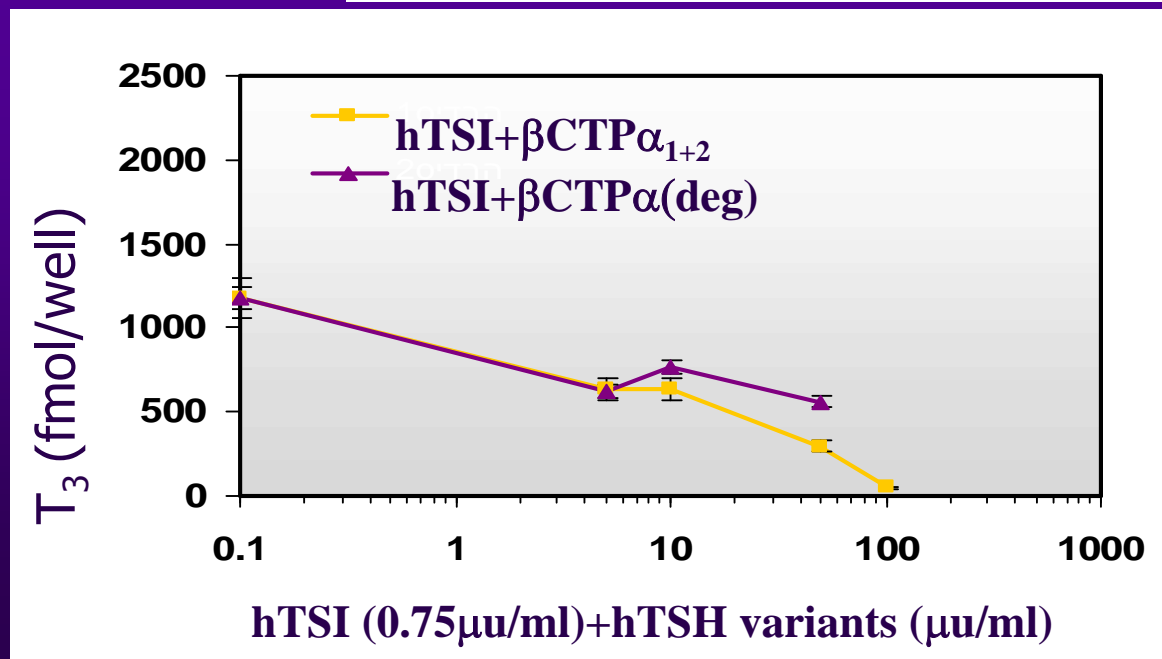
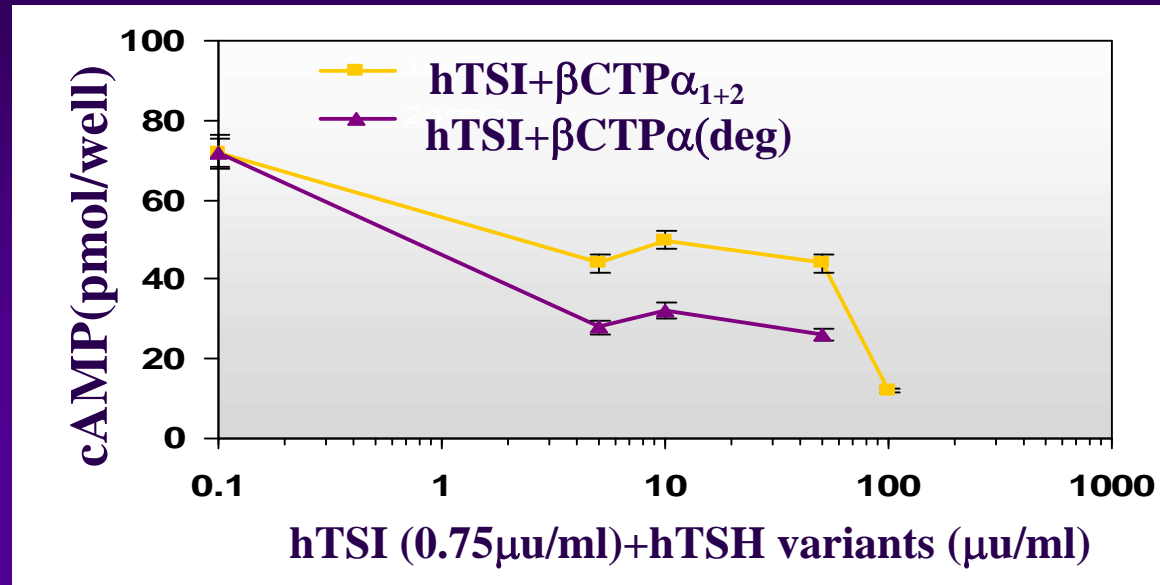


IC₅₀ (µU/ml)

| variant | value |
|----------------|-------|
| hTSHβCTPa1+2 | 70 |
| hTSHβCTPa(deg) | 158 |

| variant | value |
|----------------|-------|
| hTSHβCTPa1+2 | 33 |
| hTSHβCTPa(deg) | 135 |

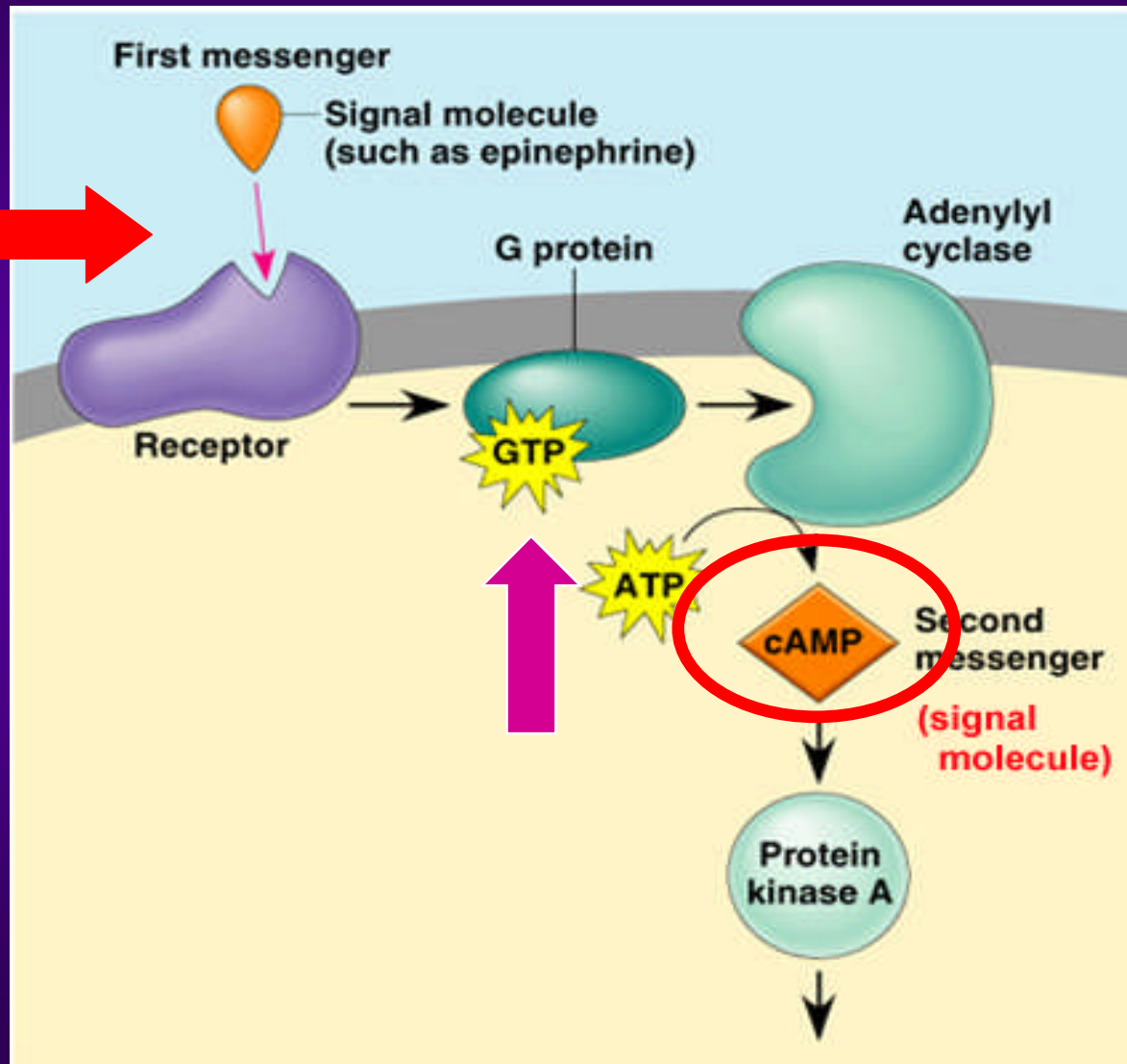
TSH Antagonist

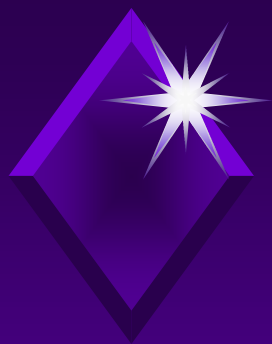




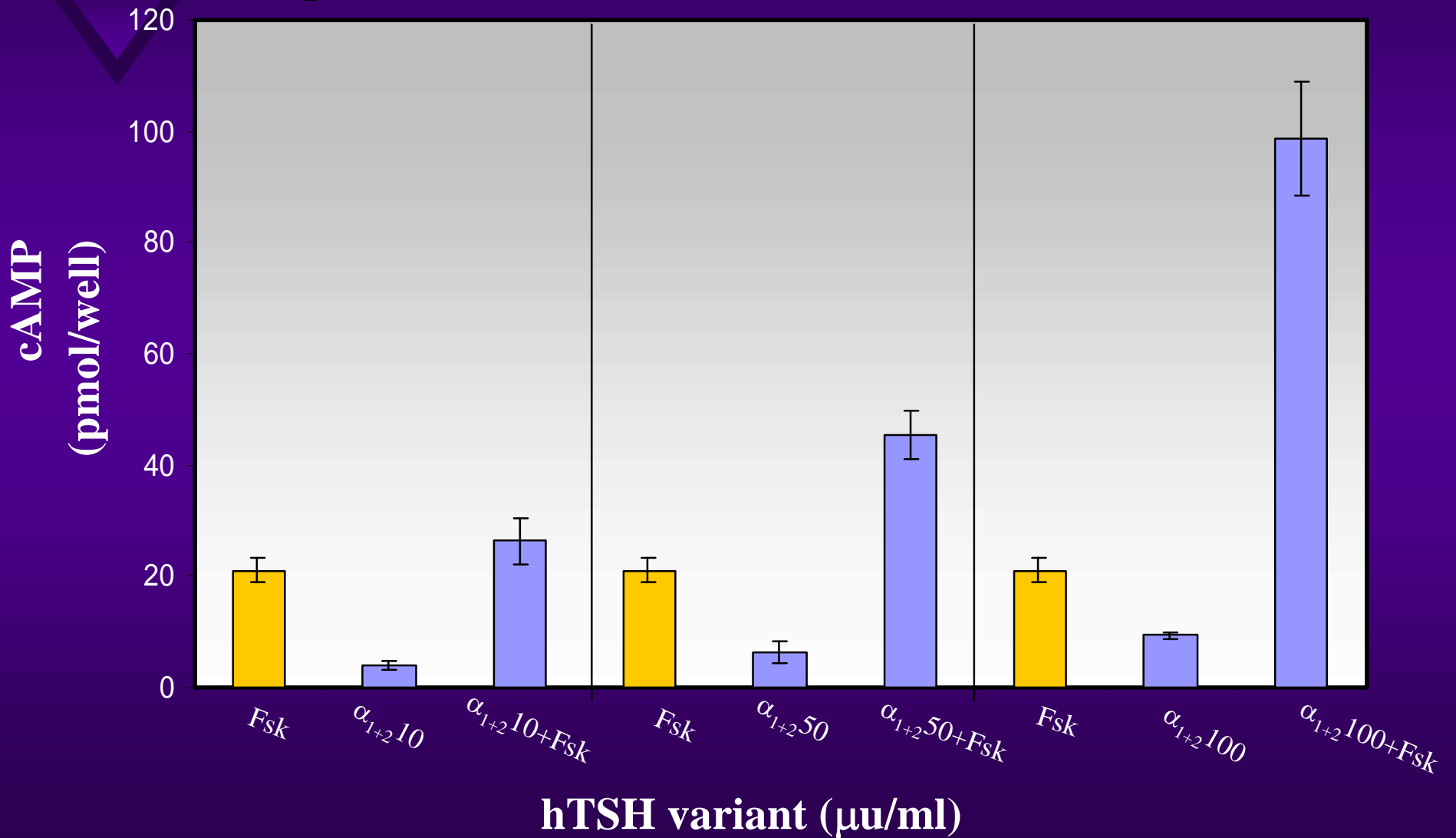
G-protein cellular responses:

hTSH/
hTSI





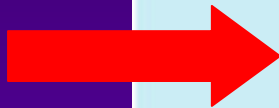
Biological activity of hTSH variants in the presence and absence of forskolin





G-protein cellular responses:

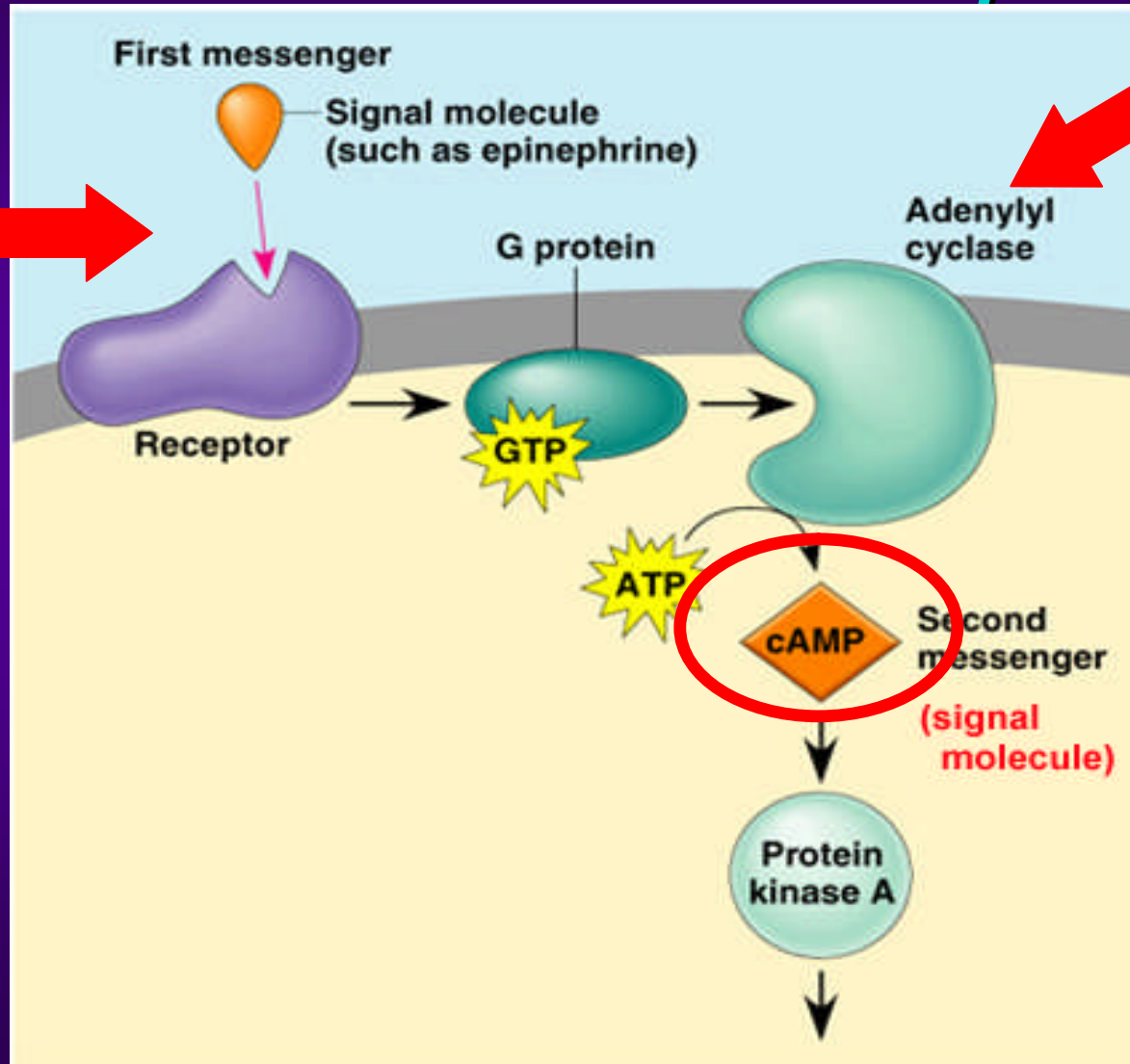
hTSH



Agonist/

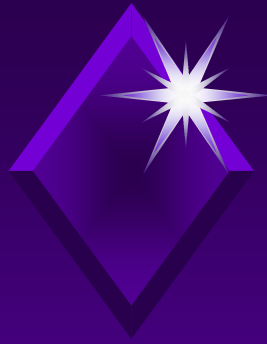
Antagonist

hTSI



forskolin



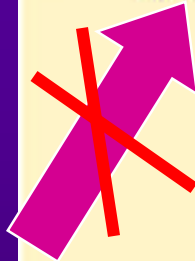


G-protein cellular responses:

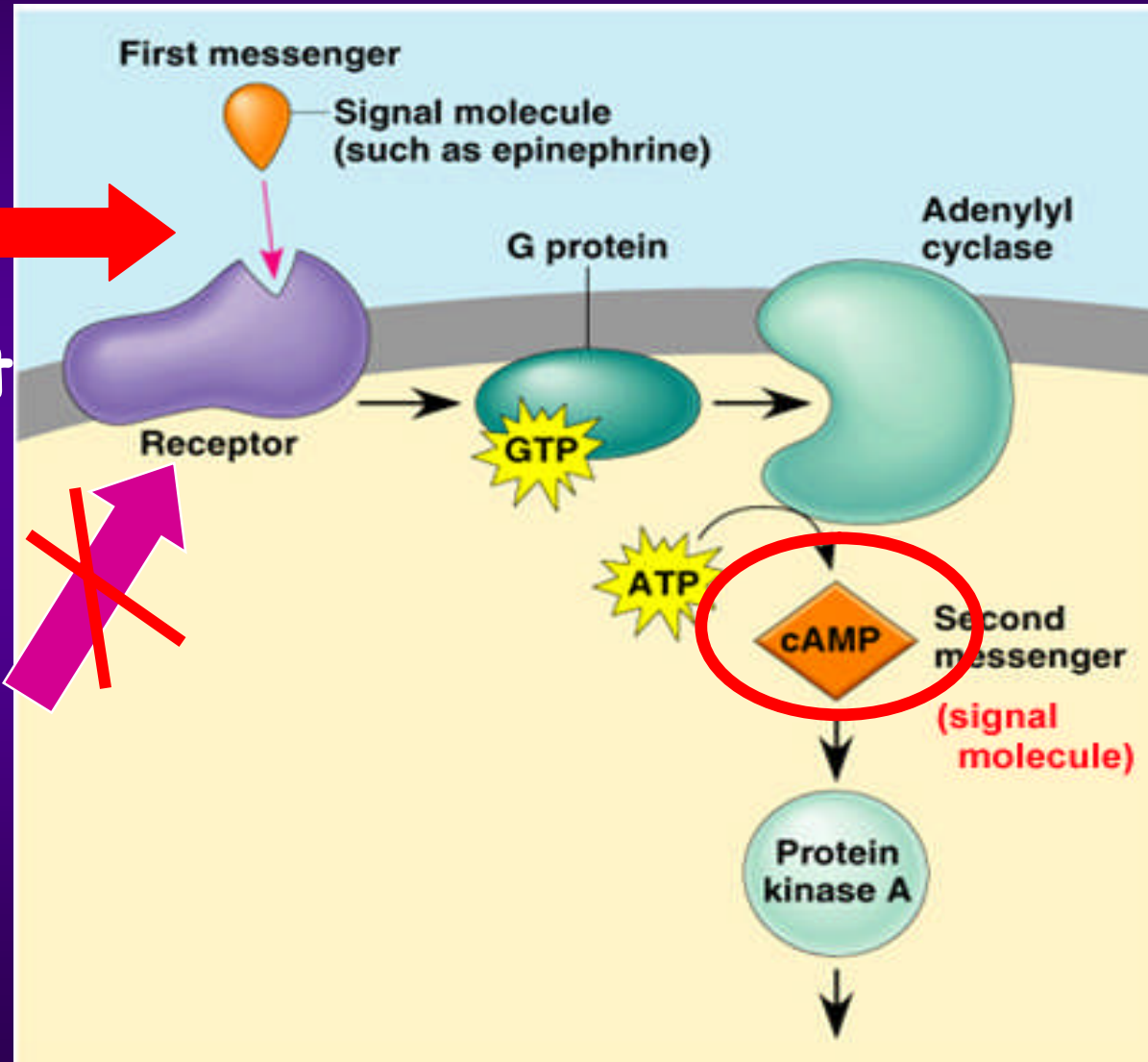
hTSH



Antagonist



hTSH
TSI

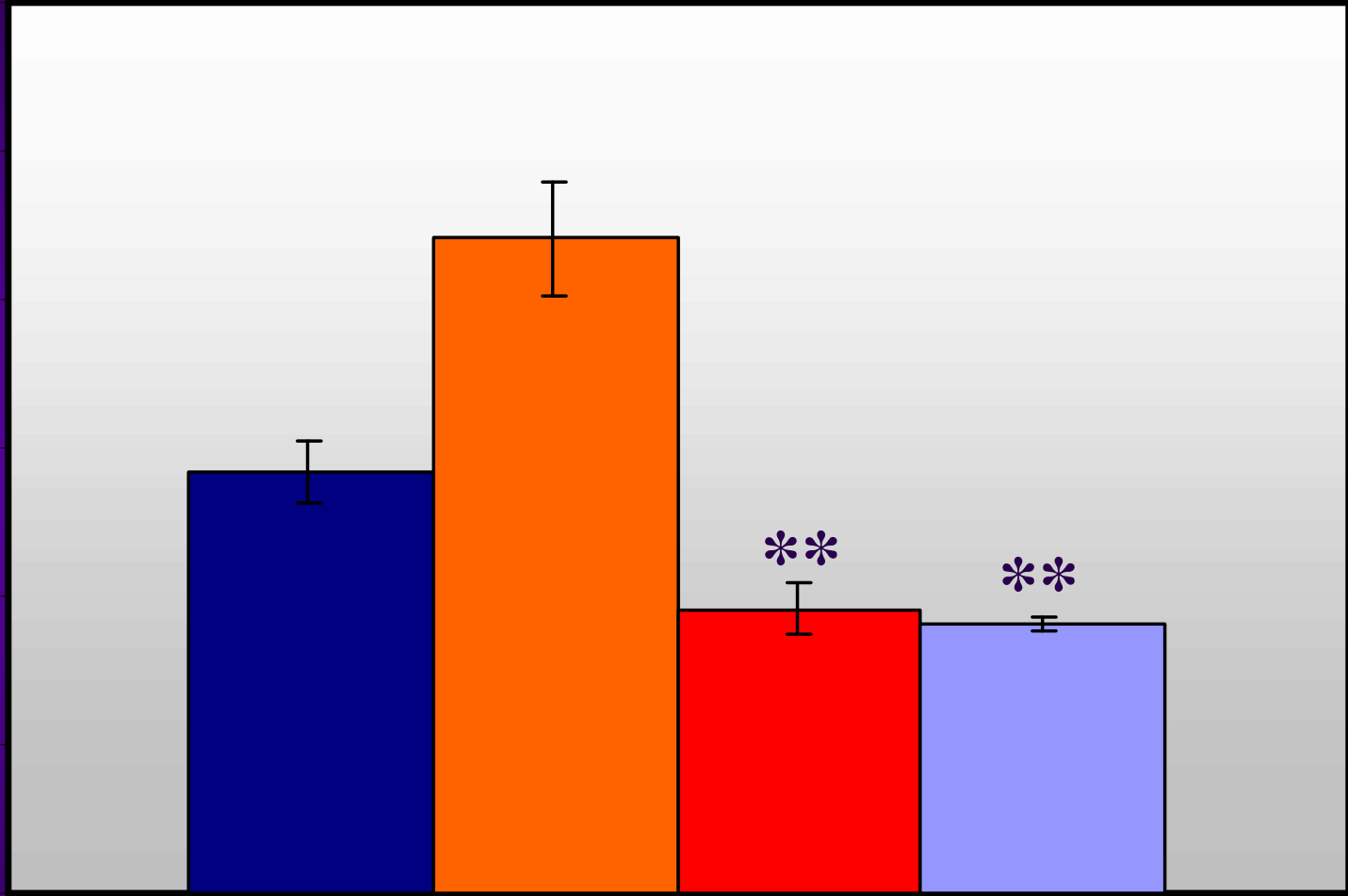


In Vivo (8h after injection)



T₄ (μg/dl)

12
10
8
6
4
2
0



bTSH

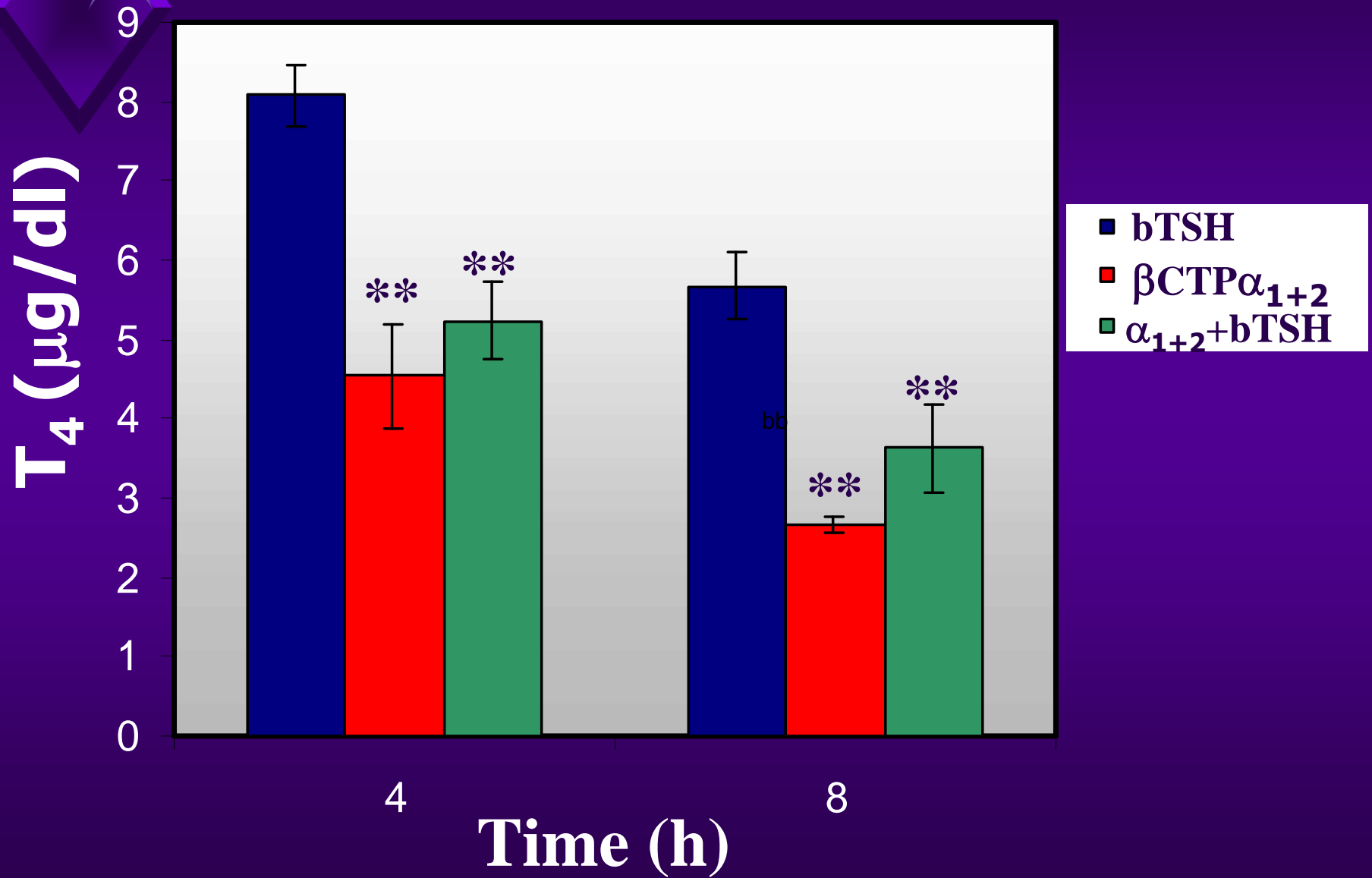
β_αWT

βCTP_αdeg

βCTP_α1+2

8

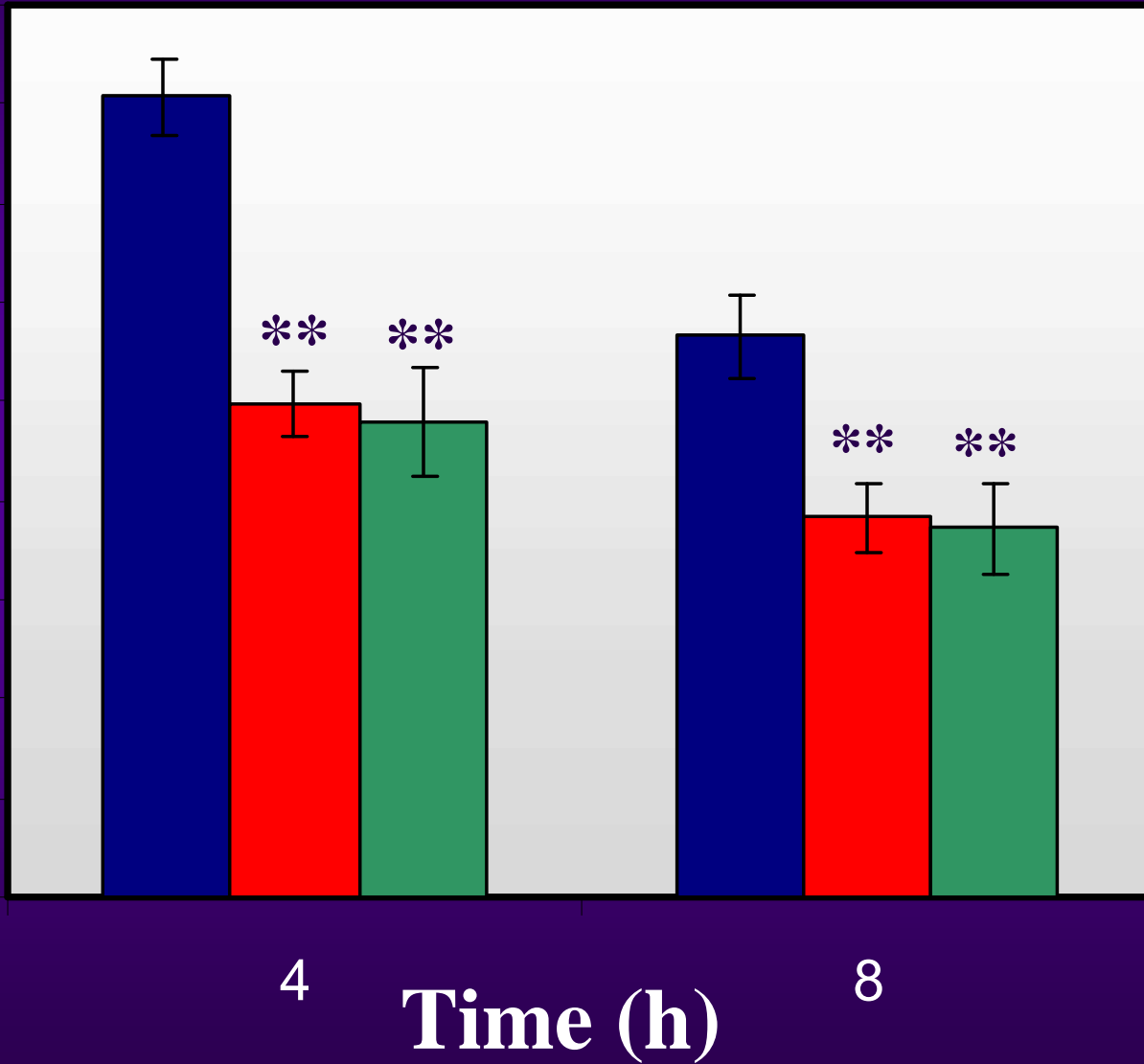
In Vivo - Competition (α_{1+2})



In Vivo – Competition (deg)

T₄ (μg/dl)

9
8
7
6
5
4
3
2
1
0



- bTSH (ch)
- βCTPα(deg)
- deg+bTSH (ch)

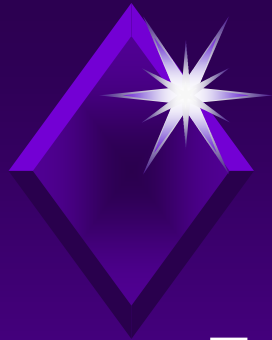


Summary

- hTSH α and β subunits, encoded as single chain, retain a biologically active conformation similar to that of the heterodimer

- The *N*-linked oligosaccharide units are not important for hTSH receptor binding.

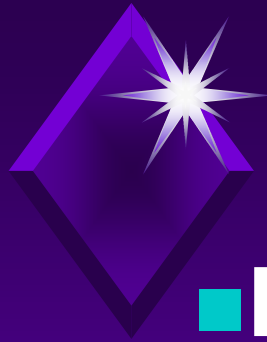
- However, *N*-linked oligosaccharide play a dominant role in signal transduction of hTSH



Conclusions

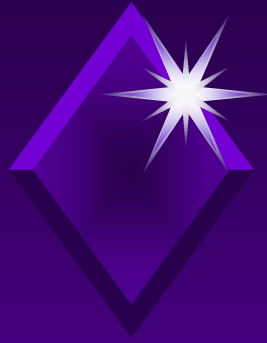
- ◆ **Ligation of the CTP cassette gene to different proteins, could be an interesting strategy for increasing the *in vivo* half-life and *in vivo* bioactivity**

This will reduce the amount of drug, and the number of injections



Conclusions

- Deletion of the *N*-linked oligosaccharides from TSH resulted in partial antagonists of TSH and TSI at the level of the receptor binding site.
- **TSH variants may offer a novel therapeutic strategy in the treatment of hyperthyroidism and Graves' disease.**

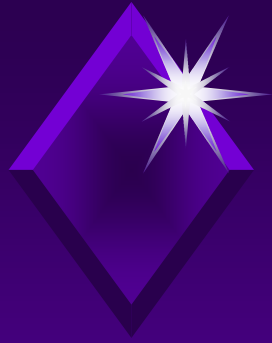


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Thank You