

# Clinical Development of Pegylated Biologics

## Science to Market

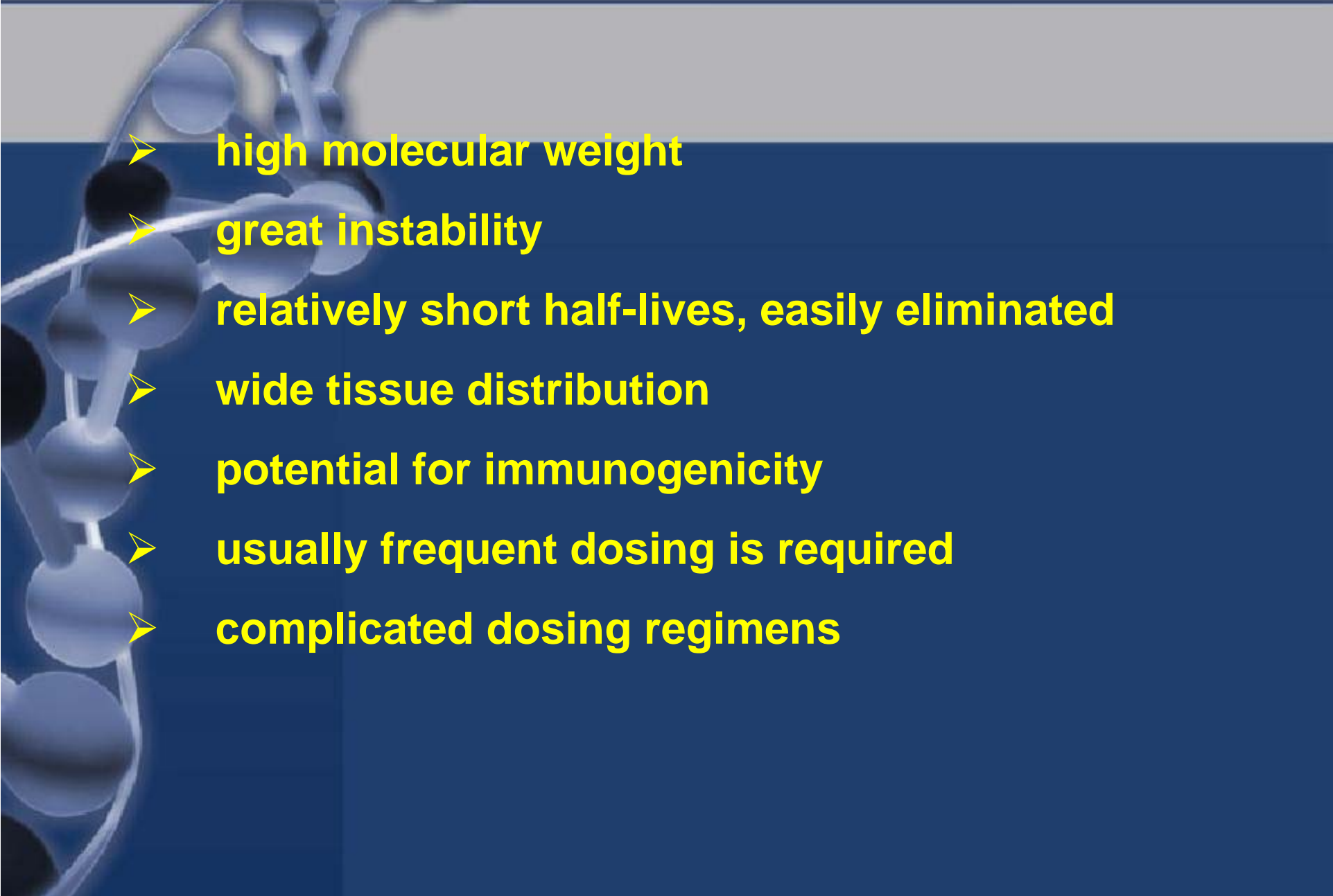
Posttranslational Modifications of Proteins -  
Consequences and Therapeutic Use

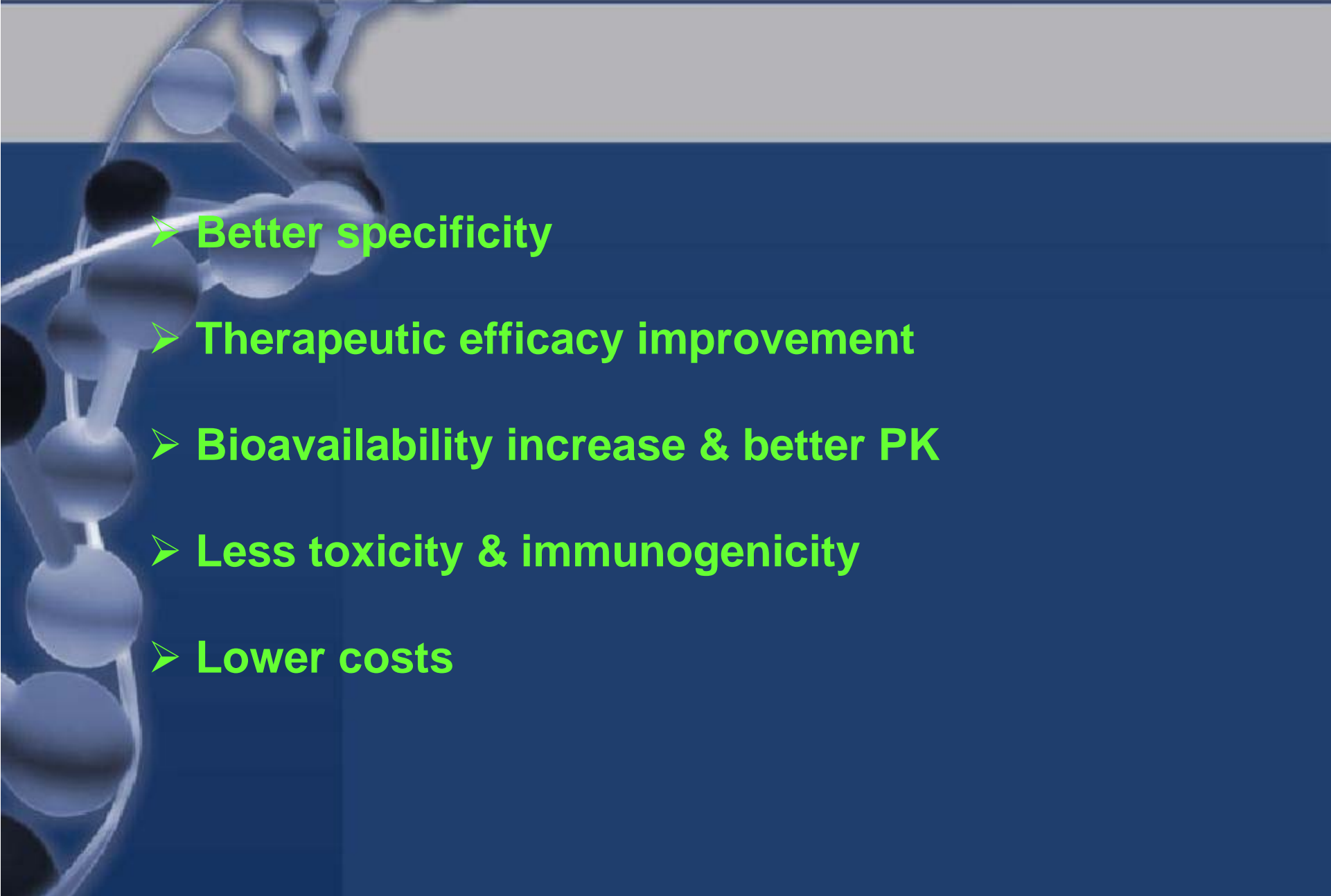
Vienna, Austria, February 24 - 25, 2010

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Pula (Cagliari), Sardinia, Italy

No.	Previous Year	Class of Products	2008 sales (US\$ bln.)	2007 sales (US\$ bln.)	Selected Branded Products
1	2	Anti-TNF Antibodies	16.36	12.98	Enbrel, Remicade, Humira, <u>Cimzia</u>
2	1	Major Cancer Antibodies	15.59	15.74	Rituxan/MabThera, Herceptin, Avastin, Erbitux, Vectibix
3	4	Insulin and Insulin Analogs	10.90	11.19	Humalog, Humulin, Lantus, Levemir, Novorapid, Actrapid, Novolin
4	3	Erythropoietins	10.05	11.82	Aranesp, Procrit Eprex, Epogen, Neo-Recormon, ESPO, Dynepo, Binocrit
5	6	Interferon beta	5.35	5.35	Avonex, Rebif, Betaferon / Betaseron
6	7	G-CSF	5.18	4.82	<u>Neulasta</u> , Neupogen, Neutrogin, GRAN
7	5	Rec. Coagulation Factors	4.94	5.39	Novoseven, Kogenate, Helixate, Refacto, Advate, Recombinate, Benefix
8	10	Enzyme Replacement	2.80	2.29	Cerezyme, Fabrazyme, Aldurazyme, Myozyme, Replagal, Naglazyme, Elaprase
9	8	Human Growth Hormone	2.68	2.77	Genotropin, Norditropin, Humatrope, Nutropin, Saizen, Serostim, Omnitrope
10	9	Interferon alpha	2.56	2.74	<u>Pegasys</u> , <u>Peg-Intron</u> , Intron A
11	11	Ophthalmic Antibody	1.76	1.36	Lucentis
12	13	Antiviral antibody	1.23	> 1.13	Synagis
13	12	Follicle Stimulating Hormone	1.16	~ 1.28	Gonal-f, Puregon/Follistim
		<b>Total:</b>	<b>80.56</b>	<b>78.86</b>	

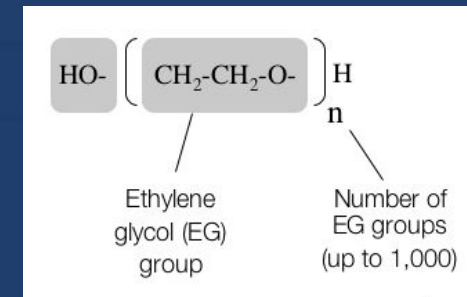
- 
- **high molecular weight**
  - **great instability**
  - **relatively short half-lives, easily eliminated**
  - **wide tissue distribution**
  - **potential for immunogenicity**
  - **usually frequent dosing is required**
  - **complicated dosing regimens**

- 
- **Better specificity**
  - **Therapeutic efficacy improvement**
  - **Bioavailability increase & better PK**
  - **Less toxicity & immunogenicity**
  - **Lower costs**

PEGylation is the covalent attachment of polyethylene glycol (PEG) to proteins.

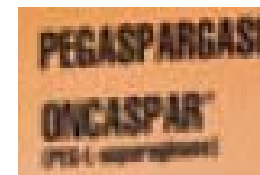
**PEG is:**

- a polyether
- chemically inert and non-toxic
- non immunogenic
- approved by Health Authorities (e.g. FDA, EMEA)
- highly soluble in water and many organic solvents
- readily cleared from the body



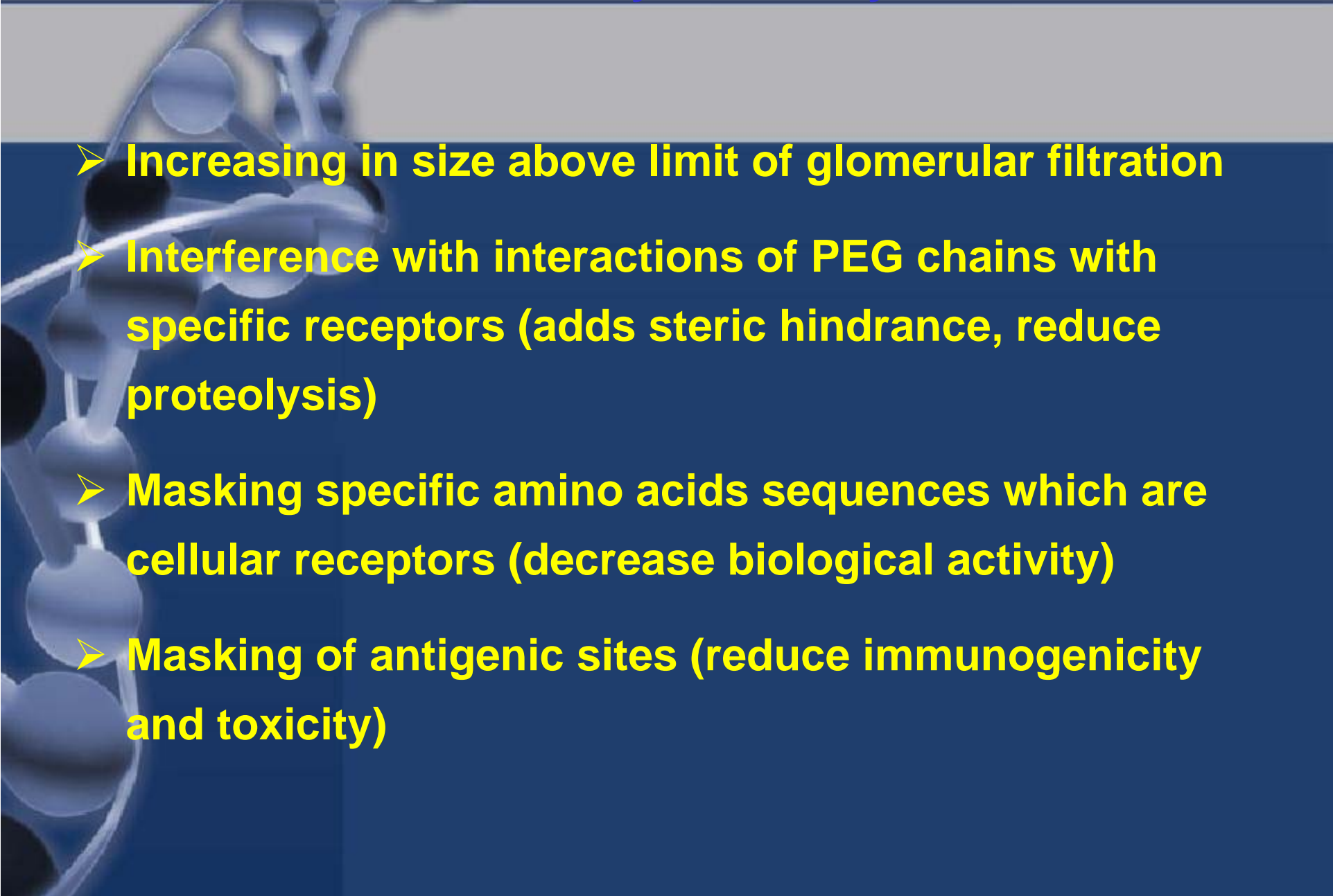
- 
- **Improve stability and solubility**
  - **Reduce immunogenicity and proteolysis**
  - **Slow clearance from the body, less frequent dosing**
  - **Improve clinical effects**
  - **Prolong patent protection**
  - **More competitive in market with an increase commercial opportunity**
  - **PEGylated proteins are marketed since 1990**

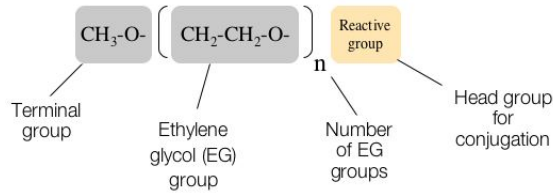
## PEGylated Protein in the Market



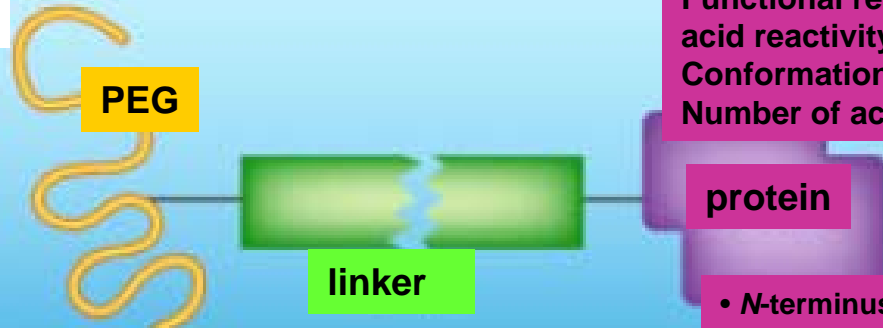
**PEGylated proteins are already on the market and more than 40 are currently under development**

Compound	Name	Status	Indication	Company
PEG-adenosine deaminase	Adagen	1990	SCID	Enzon
PEG-asparaginase	Oncaspar	1994	Hepatocellular carcinoma	Enzon
PEG-alpha-interferon 2b	PEG-Intron	2000	Hepatitis	Schering P
PEG-alpha-interferon 2a	PEGASYS	2001/2002	Hepatitis	Roche
PEG-hGR	Pegvisomant	2002	Acromegaly	Pfizer
PEG-filgrastim	Neulasta	2002	Chemotherapy-induced neutropenia	Amgen
PEG-anti-TNF Fab	CIMZIA	2008	Rheumatoid arthritis	UCB
PEG-erythropoietin	CERA	2008	Chronic renal anaemia	Roche

- 
- **Increasing in size above limit of glomerular filtration**
  - **Interference with interactions of PEG chains with specific receptors (adds steric hindrance, reduce proteolysis)**
  - **Masking specific amino acids sequences which are cellular receptors (decrease biological activity)**
  - **Masking of antigenic sites (reduce immunogenicity and toxicity)**



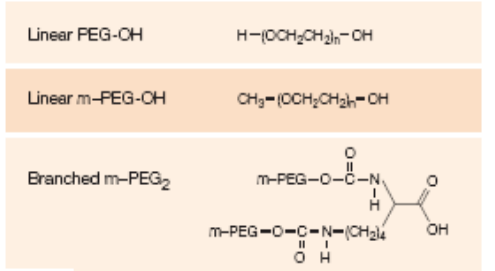
Different MW  
Linear  
Branched



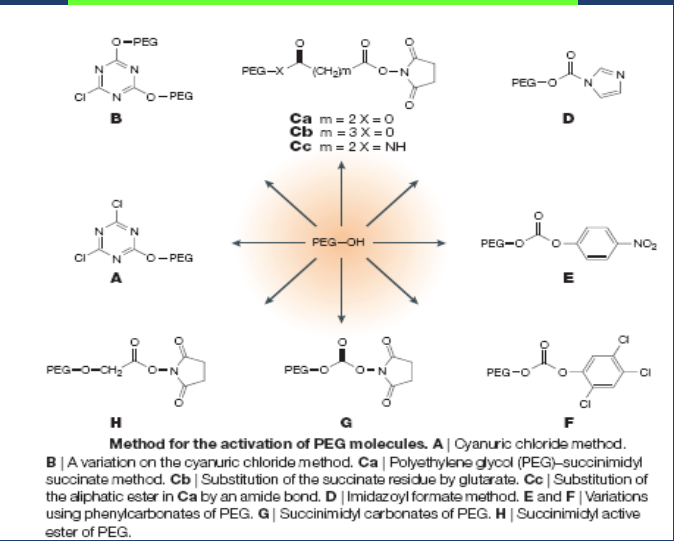
Functional reactive groups (amino acid reactivity)  
Conformation and PEG accessibility  
Number of active reactant groups

- N-terminus (NH<sub>2</sub>) site specific
- Cys (SH) site specific
- Lys (NH<sub>2</sub>)
- His (imidazole NH), Arg (guanidinium).
- C-terminus(CO<sub>2</sub><sup>-</sup>), Asp(CO<sub>2</sub><sup>-</sup>), Glu(CO<sub>2</sub><sup>-</sup>).
- Tyr (OH), Ser (OH) and Thr (OH).

Different size and composition  
Functional reactive groups  
Selectivity (site specific reaction)  
Labile bound/linkage



Structural formulae of polyethylene glycol (PEG) molecules. m-PEG, monomethoxy-PEG.



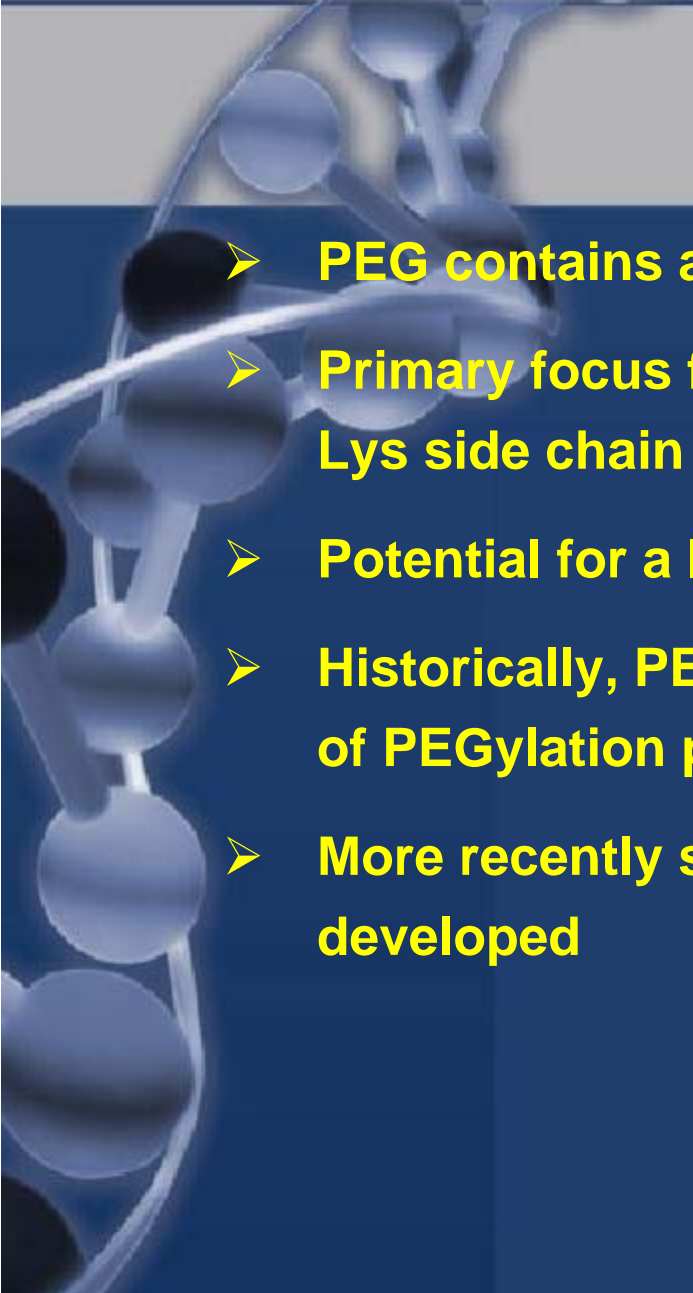
## 1. Chemical

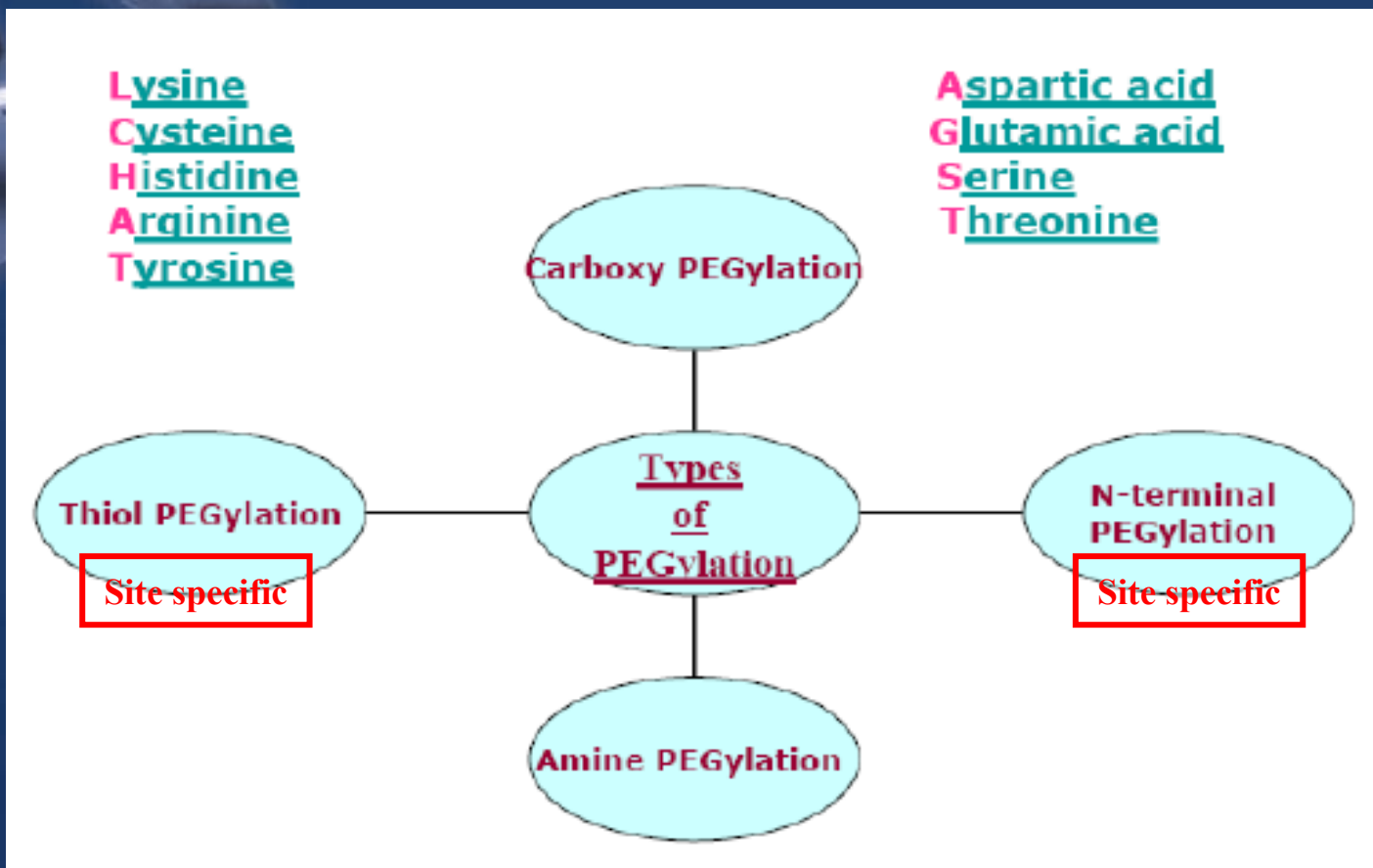
- Easy technology
- 1° generation produces random multi-PEGylated products
- 2° generation produces site specific mono-PEGylated products (SH, N-terminal, etc.)
- Needs of accurate purification process due to the large impurity amount (non specific PEGylation by-products)

## 2. Enzymatic

- Can be a simple technology
- Site specific monoPEGylation
- The PEGylation is site-selective due both to specific aminoacid (and sequence) and structural conformation
- Low impurity content and high yield

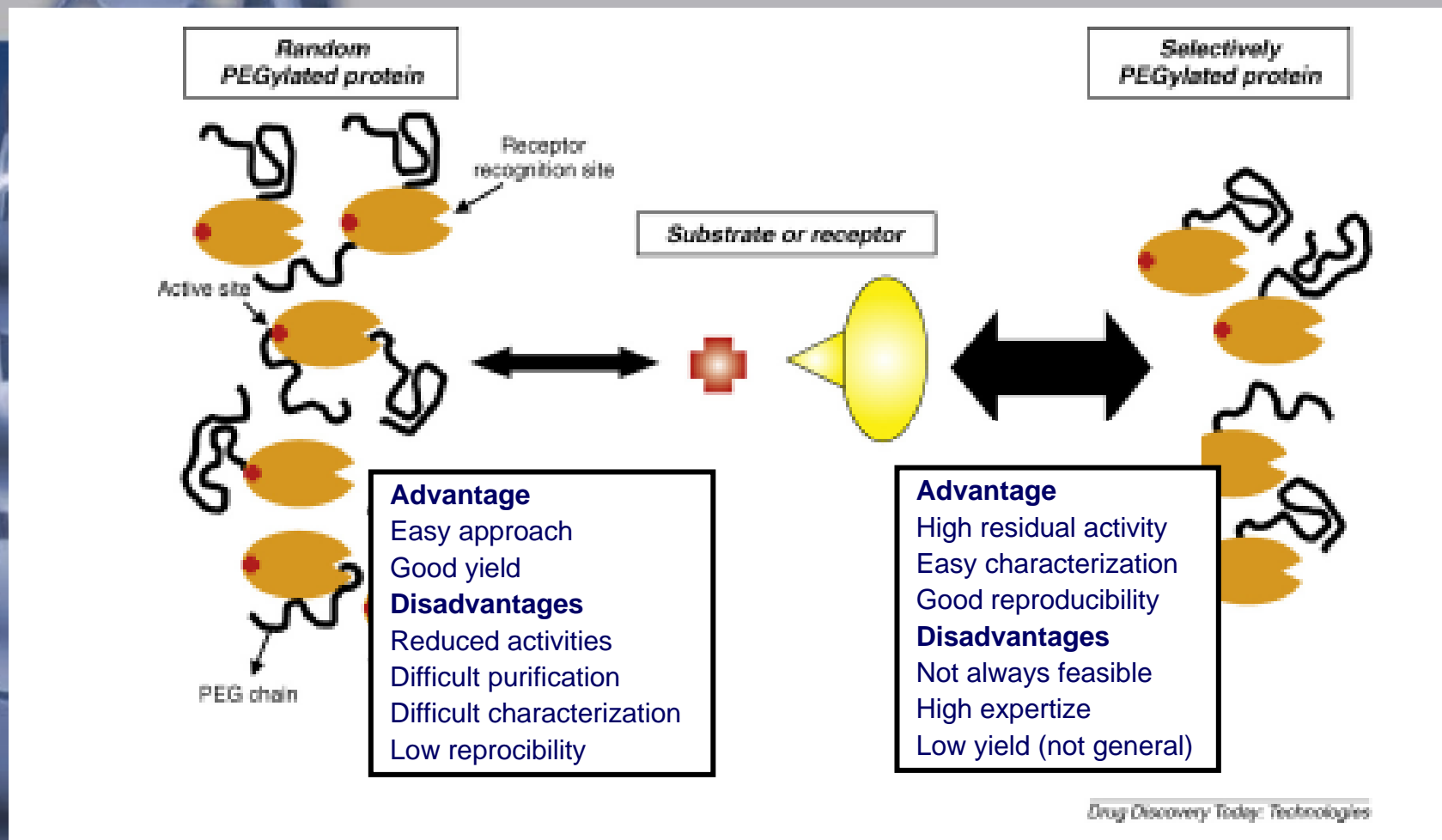
## 3. Mutant protein PEGylation

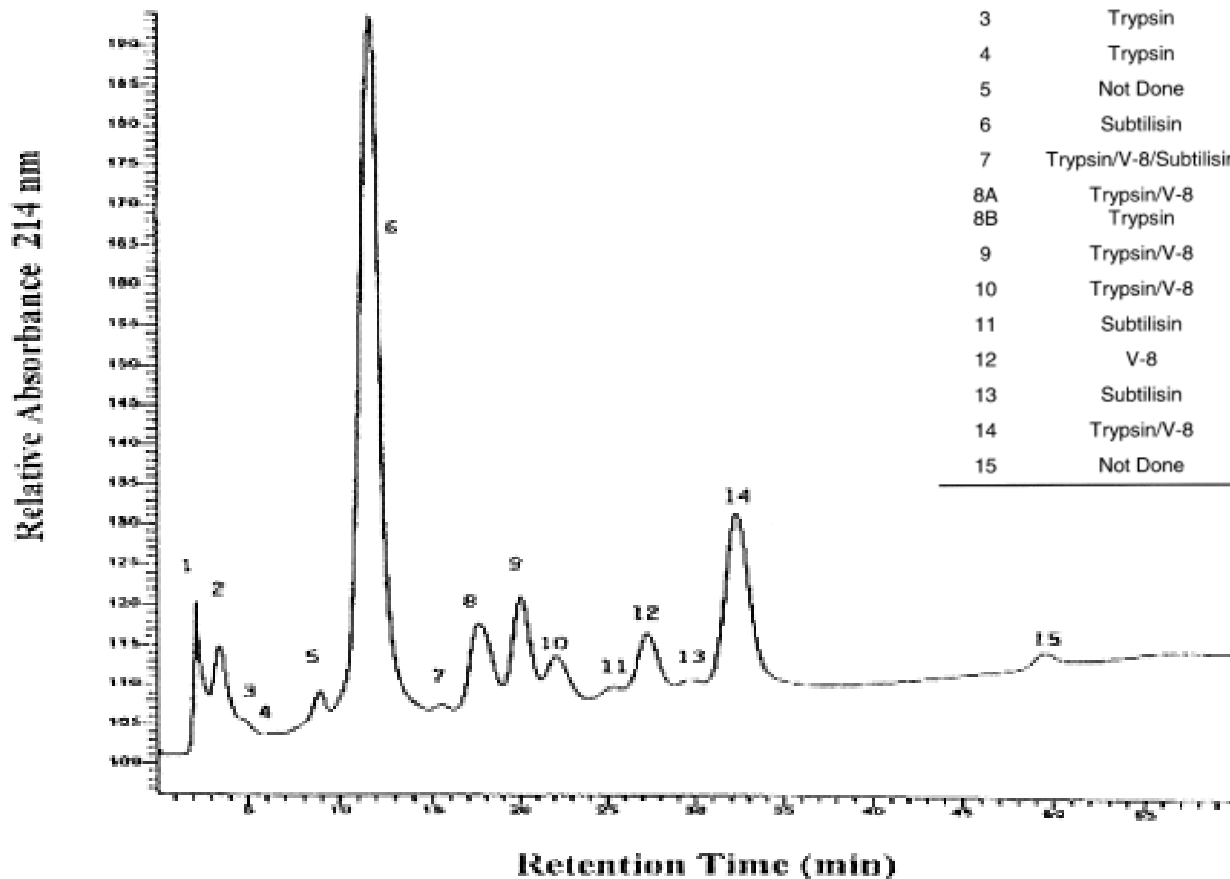
- 
- **PEG contains a chemical group reactive toward protein.**
  - **Primary focus for conjugation has been the N-terminal and Lys side chain amino groups.**
  - **Potential for a large number of positional isomers.**
  - **Historically, PEGylation results in a heterogeneous mixture of PEGylation products.**
  - **More recently site specific PEGylation have been developed**



Comparison between PK and PD PEGylated and the native protein

PEGylated-drugs	PK Half-life ( $t_{1/2}$ ) (h)		PD <i>In vitro</i> activity	Species
	Parent drug ( $t_{1/2}$ ) (h)	PEGylated drug ( $t_{1/2}$ ) (h)	% Activity retained	
PEG-arginine deiminase	2.8	50	48	rats
PEG-Catalase	1	10	95	mice
PEG-Methioninase	2	38	70	primates
PEG-Superoxide dismutase	0.01	38	51	mice
PEG-Uricase	3	72	100	human
PEG-Tricosanthin	3.3	8.3	14	rats
PEG-Lysostaphin	5	>>25	57	mice
PEG-GCSF	1.8	7	41	rats
PEG-IFN- $\alpha$ 2a	0.7	51	7	mice
PEG-IFN- $\beta$ 1a	0.98	13	50	rats
PEG-IFN- $\beta$ 1b	1.1	16.3	50	rats
PEG-IL6	0.05	48	51	rats
PEG-TNF $\alpha$	0.07	0.7	80	rats
PEG-Calcitonin	3.31	15.4	50	rats
PEG-GLP	0.04	0.56	83	mice
PEG-hGH	0.34	10	24	rats
Fab' Fragment	0.33	9.05	100	rats
PEGvisomant	0.50	100	22	rats
Anti-VEGF RNA aptamer	24	94	25	primates

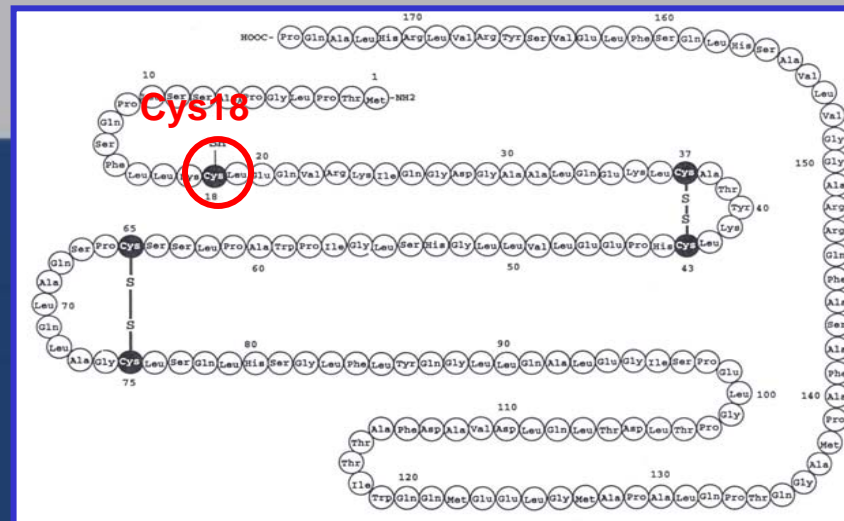




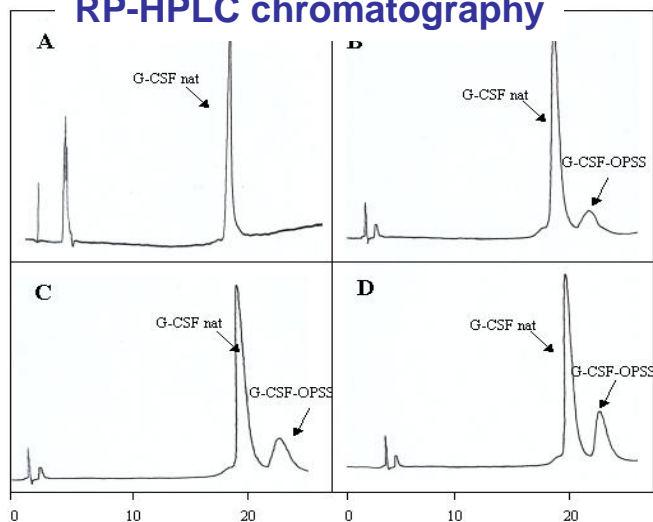
Peak #	Proteolytic Enzyme	Pegylation Site	Relative Bioactivity	Chromatographic Area %
1	Not Done	Di-peg/mono-peg mixture sites unknown	7%	3.6%
2	Trypsin/reduced	K <sup>31</sup>	13%	5.7%
3	Trypsin	K <sup>121/134</sup>	25%	1.3%
4	Trypsin	K <sup>133</sup>	32%	1.5%
5	Not Done	To be determined	To be determined	1.1%
6	Subtilisin	H <sup>34</sup>	37%	47.8%
7	Trypsin/V-8/Subtilisin	Y <sup>129</sup>	23%	0.8%
8A	Trypsin/V-8	K <sup>131</sup>	15%	6.9%
8B	Trypsin	K <sup>164</sup>	6%	6.9%
9	Trypsin/V-8	K <sup>121</sup>	9%	7.3%
10	Trypsin/V-8	K <sup>83</sup>	8%	3.6%
11	Subtilisin	H <sup>7</sup>	22%	0.9%
12	V-8	K <sup>49</sup>	13%	4.5%
13	Subtilisin	S <sup>163</sup> , K <sup>112</sup>	14%	1.1%
14	Trypsin/V-8	C <sup>1</sup>	11%	13.2%
15	Not Done	Non pegylated IFN- $\alpha_{2b}$	100%	0.7%

**Demonstration of process reproducibility and robustness**

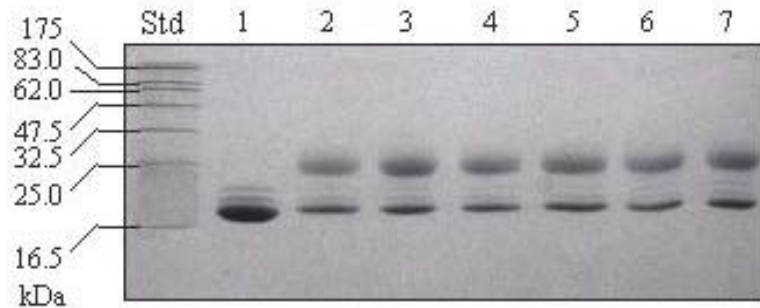
## PEG-OPSS mPEG-orthopyridyldisulfide



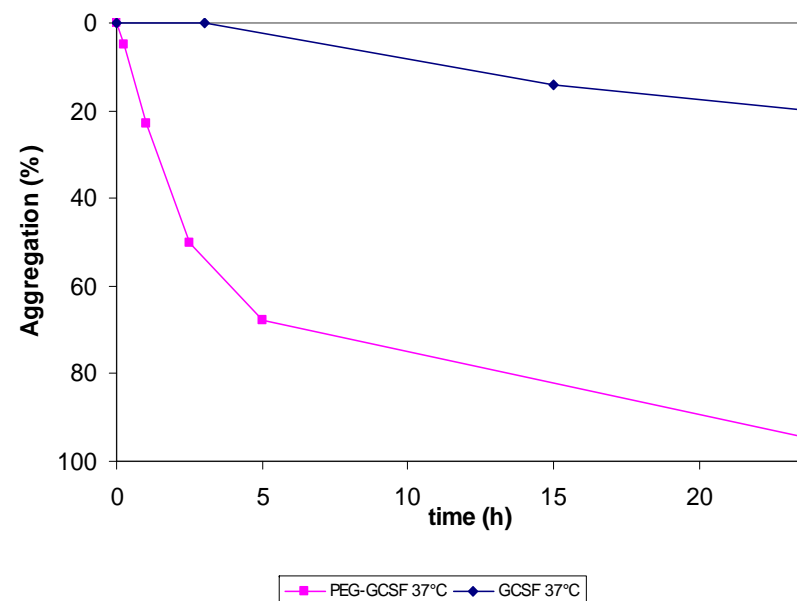
### RP-HPLC chromatography

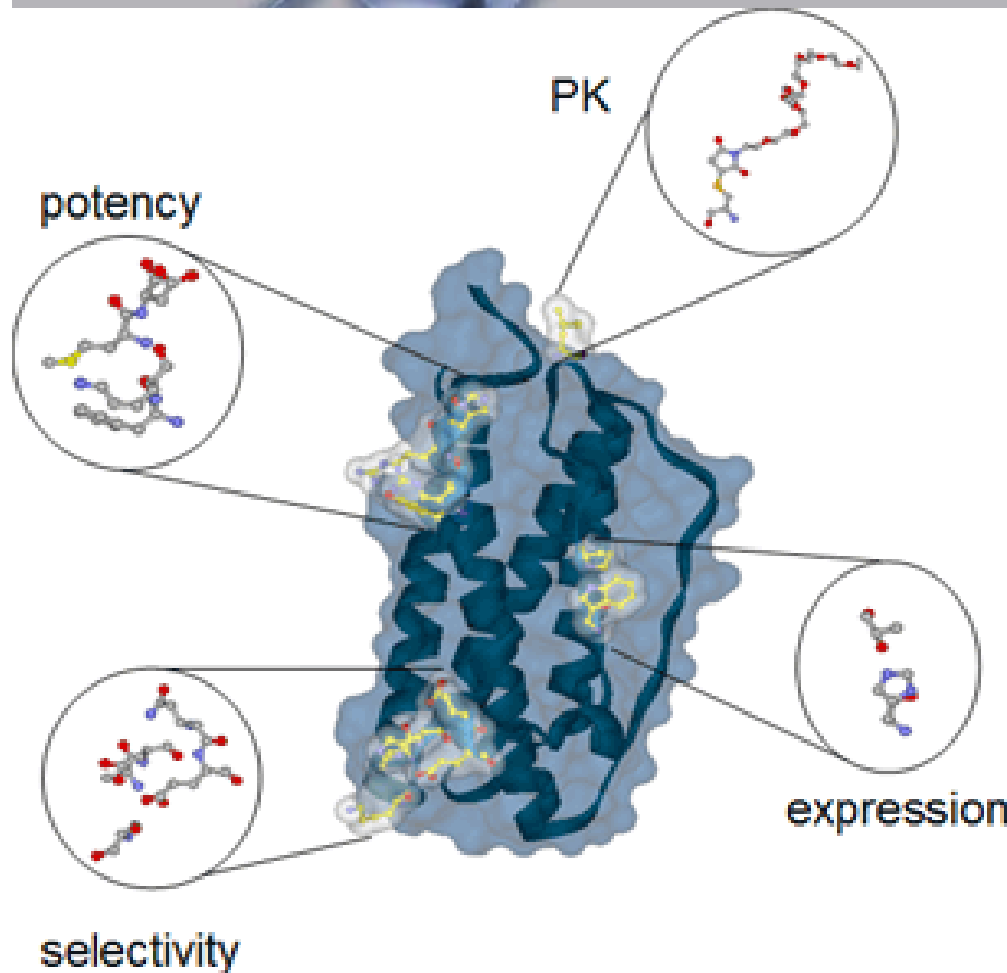


### Poliacrylamide gel electrophoresis



### Stability





**Bioinformatics and molecular design to improve the therapeutic proteins:**

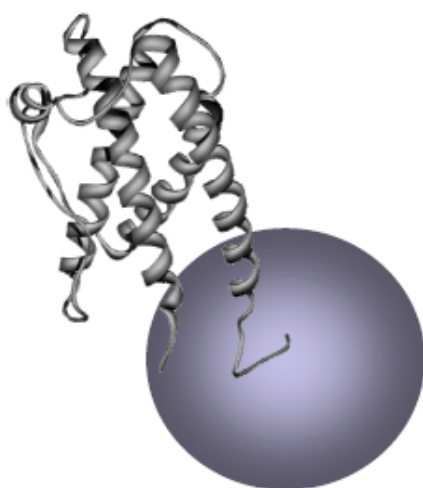
**Potency**

**Selectivity**

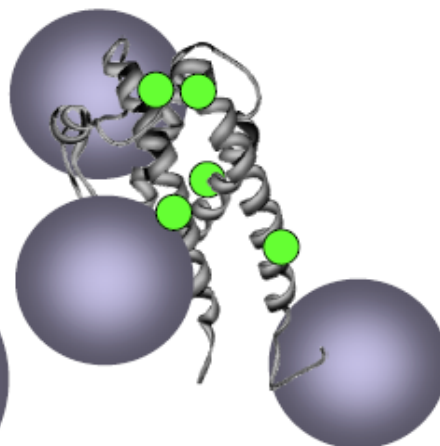
**Pharmacokinetics**

**Immunogenicity**

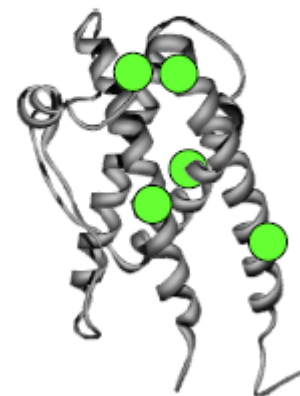
**N-terminal 20K PEG**  
**Neulasta®**



**Three 5K PEG**  
**MAXY-G34**



**MAXY-G34 Backbone**  
**Variant Human G-CSF**



AA16 **Lys -> Arg**

AA34 **Lys -> Arg**

AA40 **Lys -> Arg**

AA105 **Thr -> Lys**

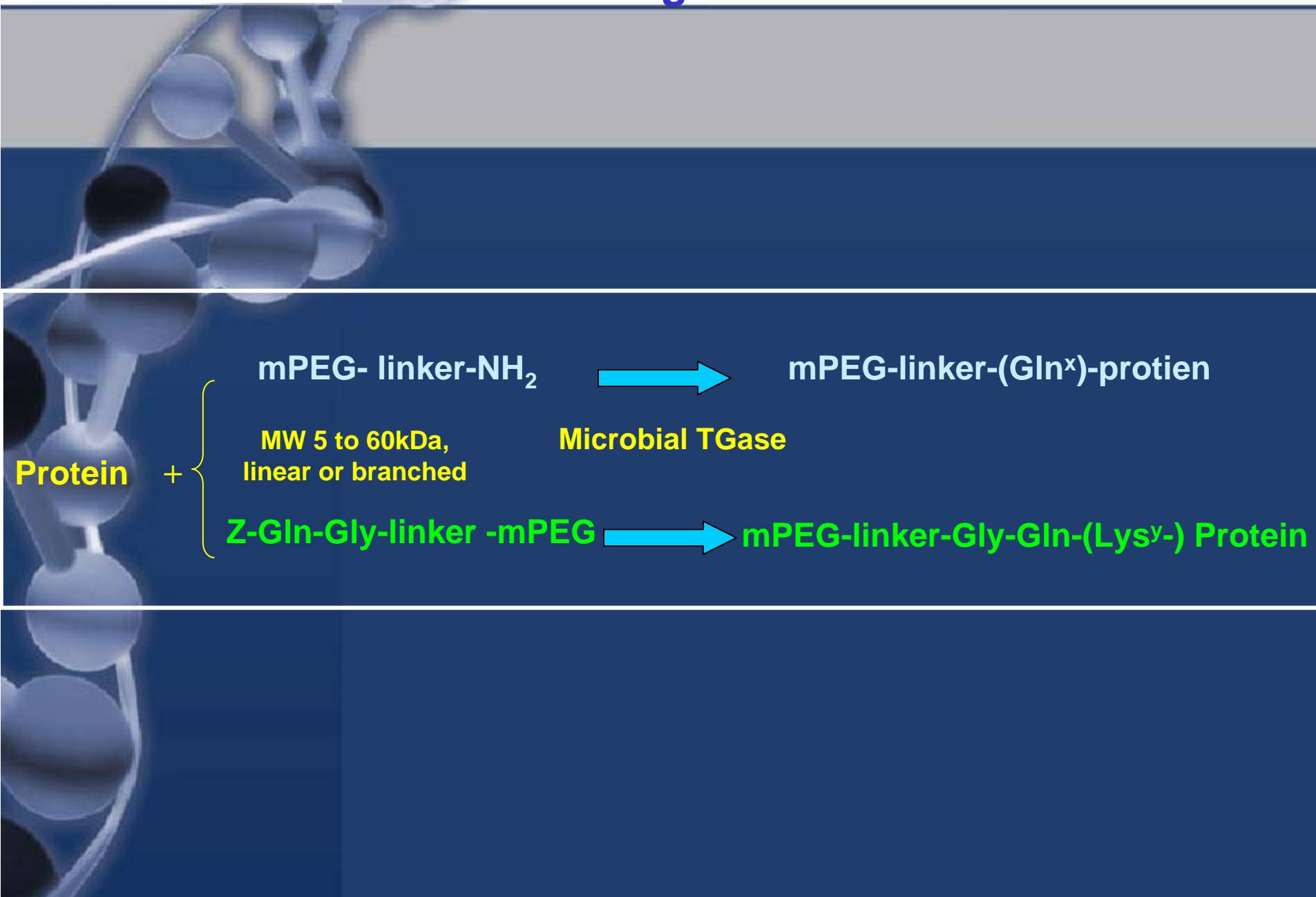
AA159 **Ser -> Lys**

## Enzymatic PEGylation

### Protein functional groups for enzymatic site specific conjugation with PEG.

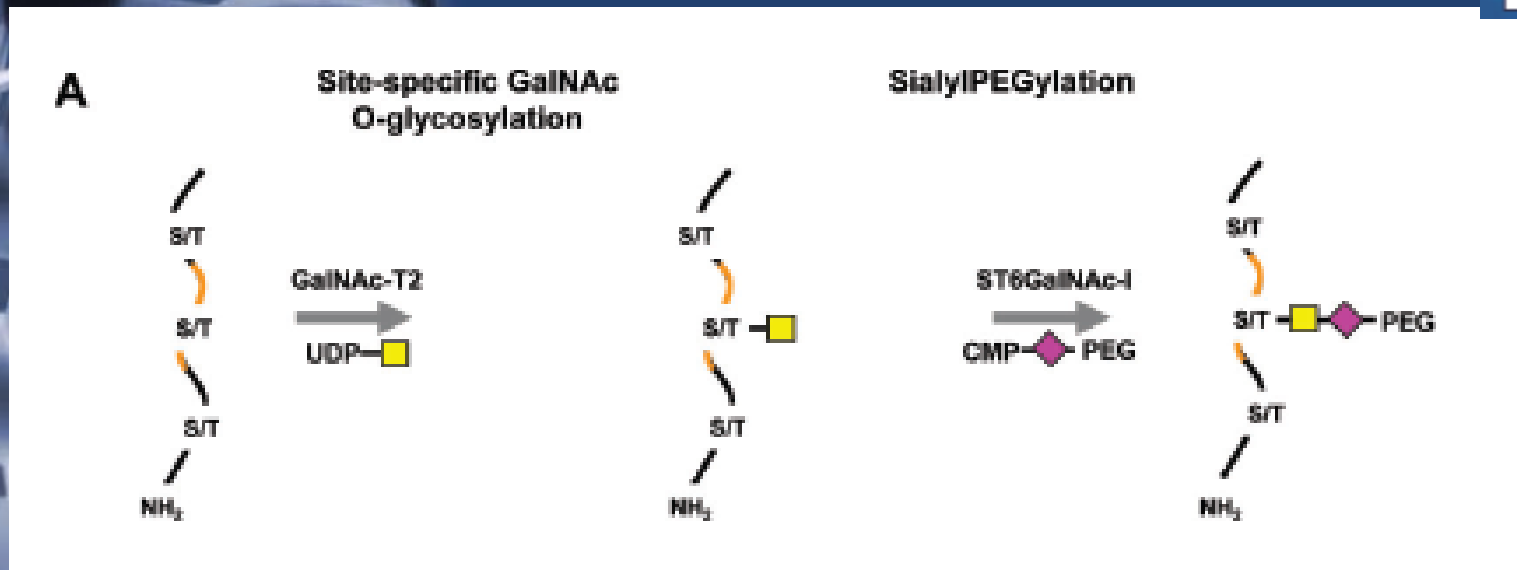
- Transgluaminase (TGase)
  - Protein (Gln<sup>X</sup>) with Peg NH<sub>2</sub>
  - Protein (Lys<sup>X</sup>-NH<sub>2</sub>) with Z-Gln-Gly-PEG
- O-glycosylation with *N*-acetylgalactosaminyltransferase (GalNAc-T2) and Sialyl PEGylation with sialyltransferase (ST6GalNAc-I)

Step 1) O- glycosylation, Protein (Ser<sup>X</sup>-OH) or (Thr<sup>X</sup>-OH) with UDP-GalNAc;  
step 2) Sialyl PEGylation, Protein Ser<sup>X</sup>/Thr<sup>X</sup>-GalNAc with CMP-PEG;



## Site-directed O-glycosylation and subsequent PEGylation at the O-glycan site with sialyltransferase.

NEOSE



Selective addition of GalNAc, using the enzyme polypeptide *N*-acetyl-galactosaminyl-transferase (GalNAc-T2) with UDP-GalNAc substrate, to a nonglycosylated recombinant polypeptides containing a single, natural unutilized O-glycosylation sites (orange), serine (S) or threonine (T), followed by PEGylation with Sialic Acid –PEG /CMP-PEG) using a sialyltransferase (ST6GalNAc-I).

## ➤ Discovery phase

- define the structure and PEGylation site,
- PEG characteristic,
- stability,
- pharmacokinetic profile
- biological efficacy and duration

## ➤ Preclinical and clinical studies

- to be discussed with EMEA through a Scientific Advice
- the regulatory requirement will be related to innovation and case by case defined (new PEGylated proteins, PEGylated proteins containing an already approved protein moiety, PEGylated proteins differing from the already approved ones by the PEG type or by the PEGylation site, biosimilar PEGylated protein)

## Conjugation site identification

- **Site-specific conjugation**



- Identification of the conjugation site
- Exclusion of positional isomers

- **Random multi-conjugation**



- Identification of the positional isomers
- Characterisation of isomers
- Demonstration of the batch to batch consistency (isomer mixture composition)

### Monographs on PEGylated –protein drug substances

- Identity by SDS PAGE, western blot
- Identity by peptide mapping
- PEGylation site definition (*not routine test, only for API characterization*)
- Purity by HPLC, (SEC, IEC, RPC)
  - Aggregates
  - Deamidates and oxidized
  - DiPEGylated and polyPEGylated
  - Residual nonPEGylated protein
- PEG-specific identity
- Residual PEG
- Potency
  - Bioassay
  - HPLC
- Endotoxins
- Bioburden

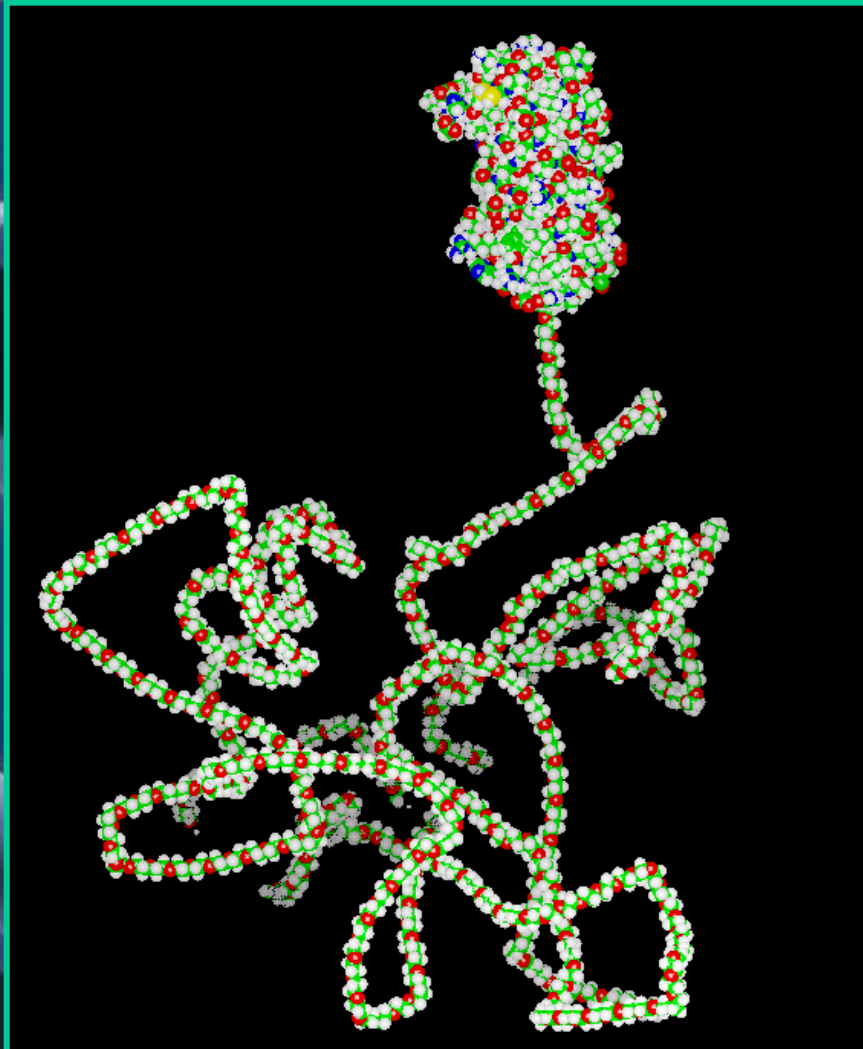


# The PEGfilgrastim

- **Grade 3 or 4 neutropenia is common**
  - risk of life-threatening infections, hospitalization, and IV antibiotics
- **Emerging data suggest other SEs associated with severe neutropenia**
- **Primary dose-limiting toxicity**
  - Chemotherapy dose delays and reductions may compromise treatment effectiveness
- **Additional impacts: economic, quality of life**

**To develop a sustained-duration form of Filgrastim which could be administered safely and which would cover a complete cycle of conventional chemotherapy with a single injection**

## N-terminal PEGyalted G-CSF



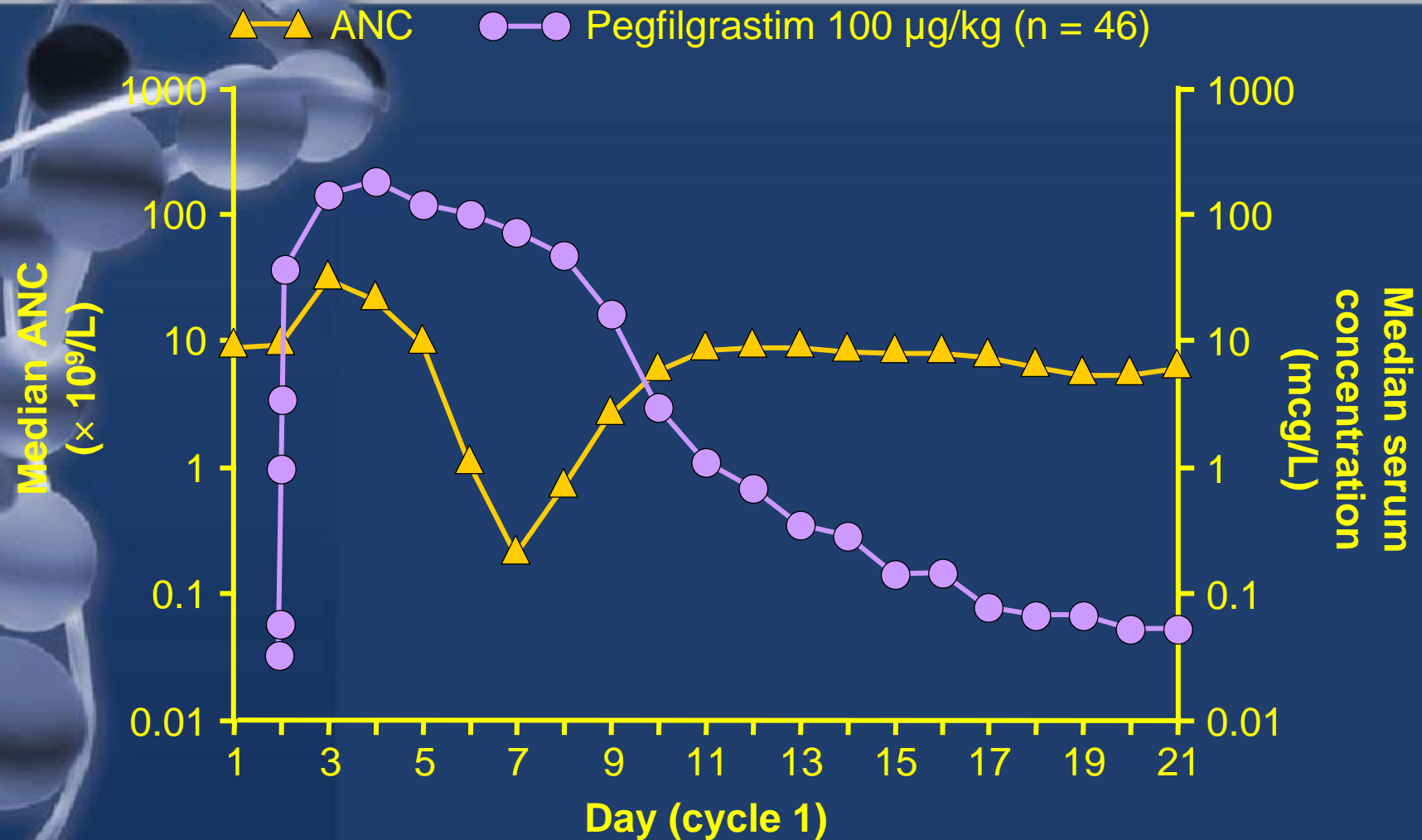
### PEG filgrastim (neulasta)

- Filgrastim core
- 20K linear PEG
- Attached to N-terminus by covalent chemistry
- resistant to renal loss
- Neutrophil-mediated regulation

### **PEGfilgrastim:**

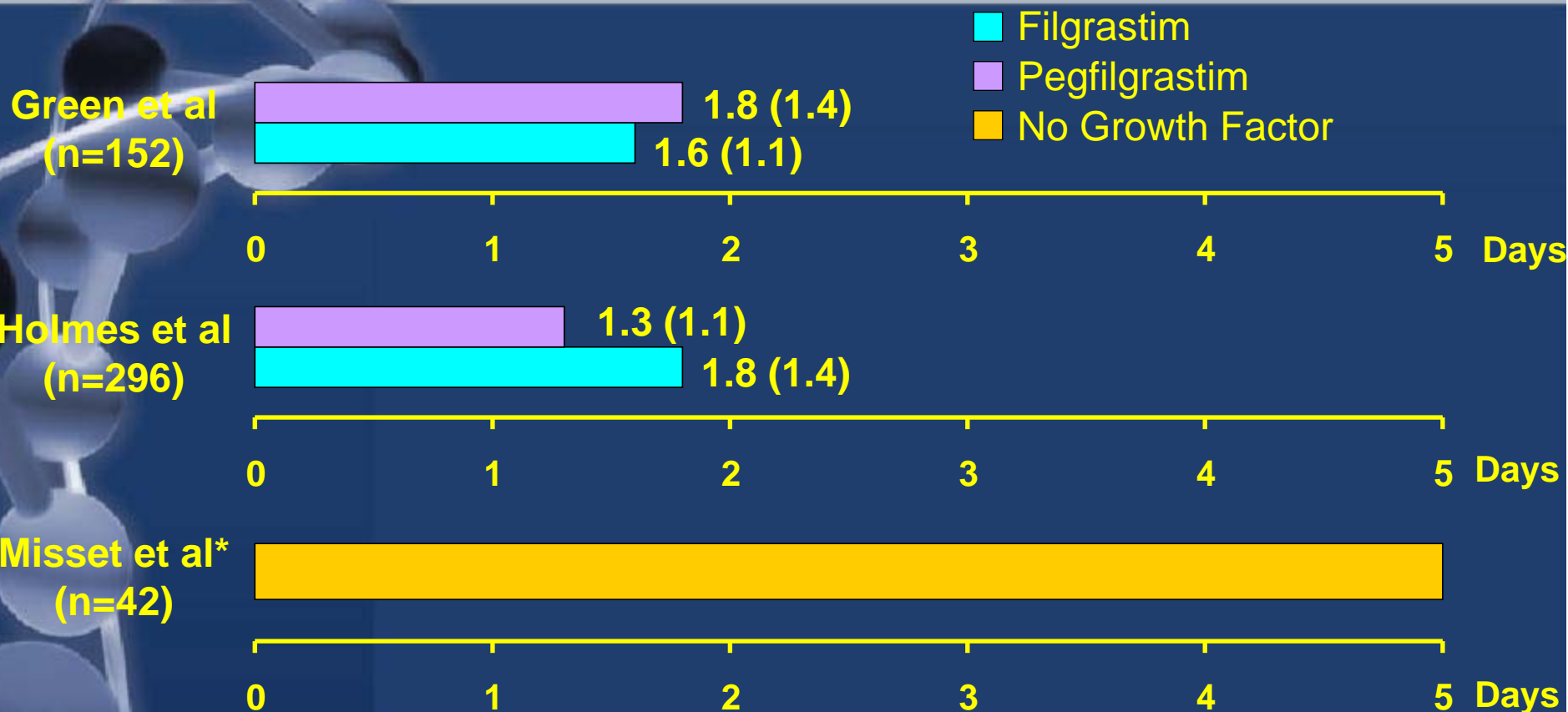
- **Has no toxicity problems**
- **Has extended half-life**
- **Produces neutrophilia in normal mice, rats, and primates**
- **Reverses therapy-induced neutropenia in mice and primates**
- **Has a “self-regulating” feature**

**Pegfilgrastim clears as neutrophil levels return to normal**



Adapted from Holmes FA, et al. *Ann Oncol.* 2002;13:903-909.

## Duration of severe neutropenia (DSN) in cycle 1



\*In patients who received a similar myelosuppressive regimen, but did not receive growth factor, the median DSN was 5 days.

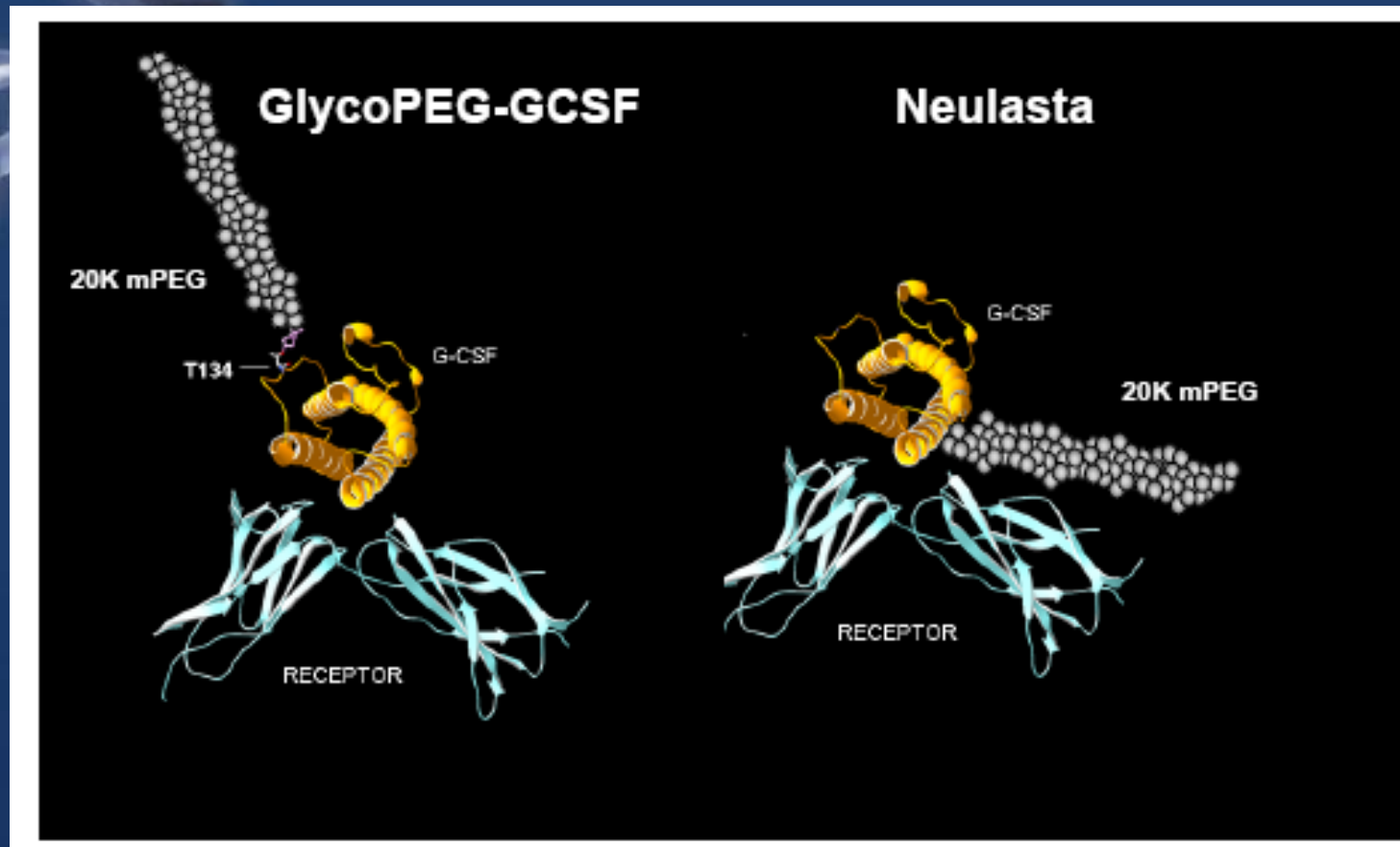
Holmes FA, et al. *J Clin Oncol.* 2002;20:727-731; Green M, et al. *Ann Oncol.* 2003;14:29-35. Misset, et al. *Ann Oncol.* 1999;10:553-560.

### Clinical demonstration of the non inferiority of PEG filgrastim versus filgrastim in the efficacy with comparative tolerability

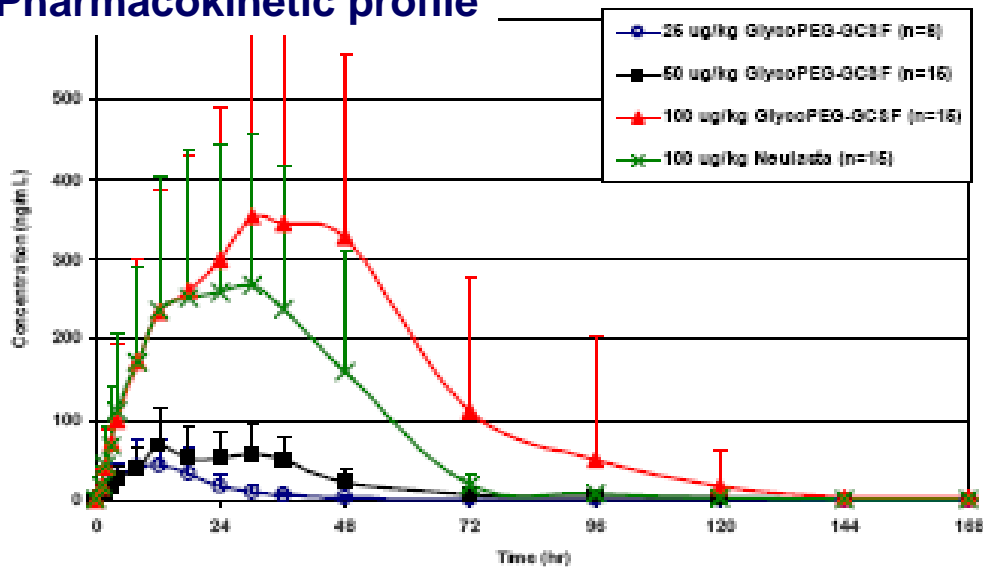
- **Single cycle administration of pegfilgrastim provides protection against neutropenic complications comparable to daily Filgrastim injections**
  - Tumor type (nonmyeloid)
  - Myelosuppressive chemotherapy regimen
  - Patient weight
- **Rate of febrile neutropenia is decreased with pegfilgrastim**
- **Fixed dose of 6-mg is effective across a wide range of body weights (46-125 kg)**

**NEOSE**

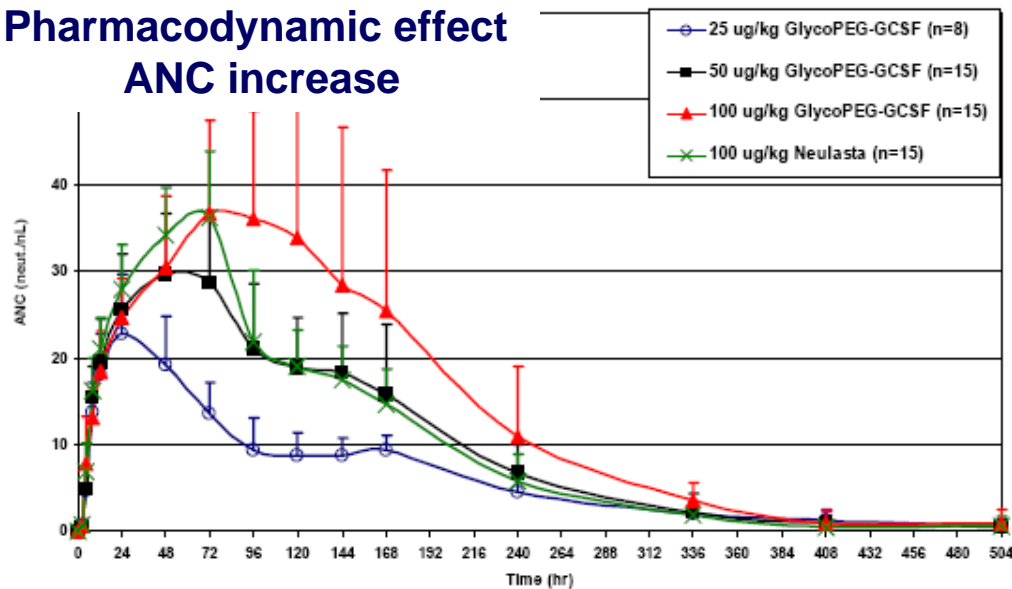
Bio  
Generix



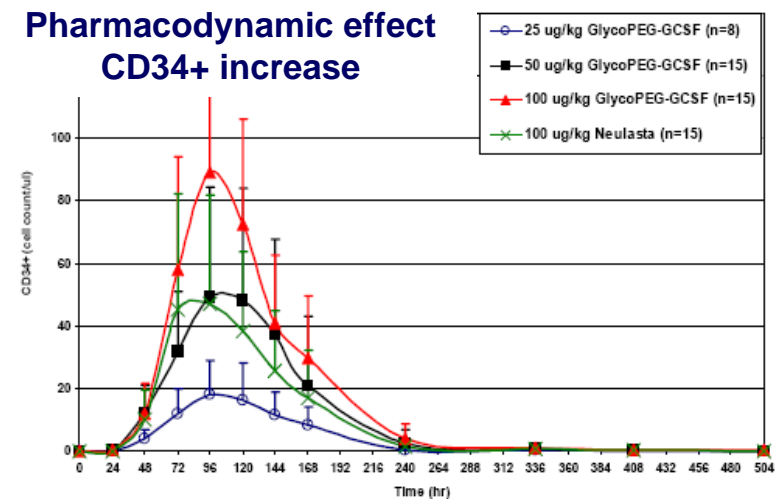
## Pharmacokinetic profile



## Pharmacodynamic effect ANC increase



## Pharmacodynamic effect CD34+ increase

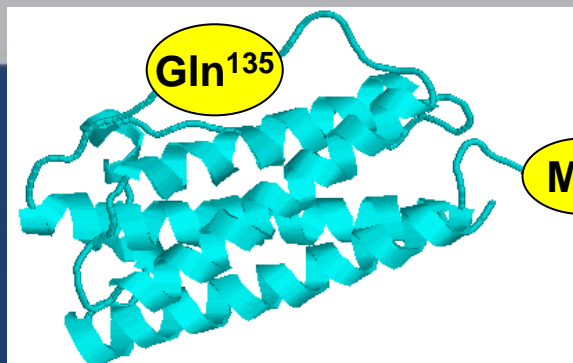




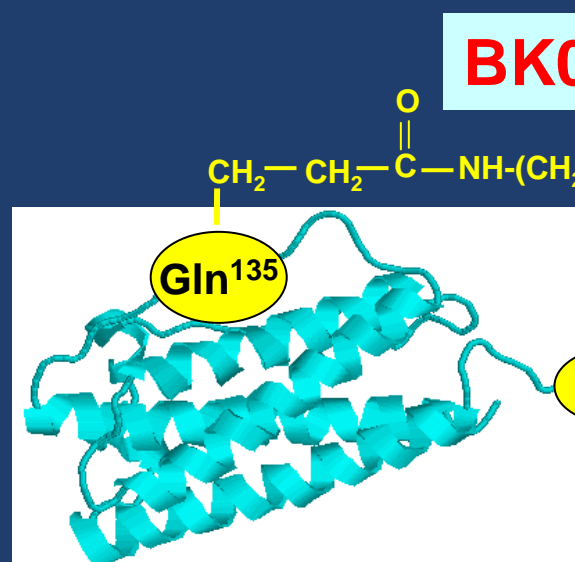
**BIO-KER**

**Met-G-CSF- Gln<sup>135</sup>-PEG20<sub>kDa</sub> (BK0026)**

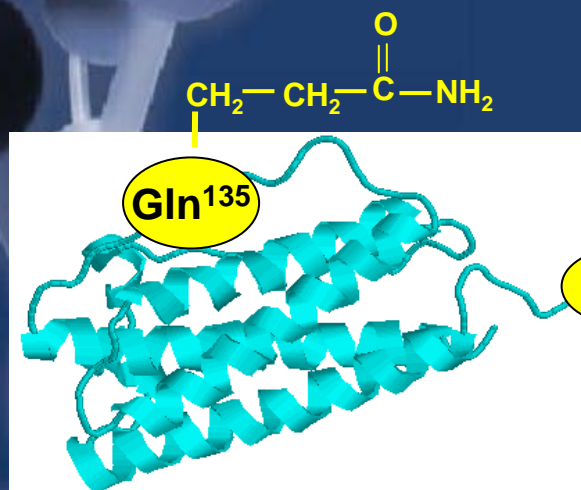
**Neulasta®**



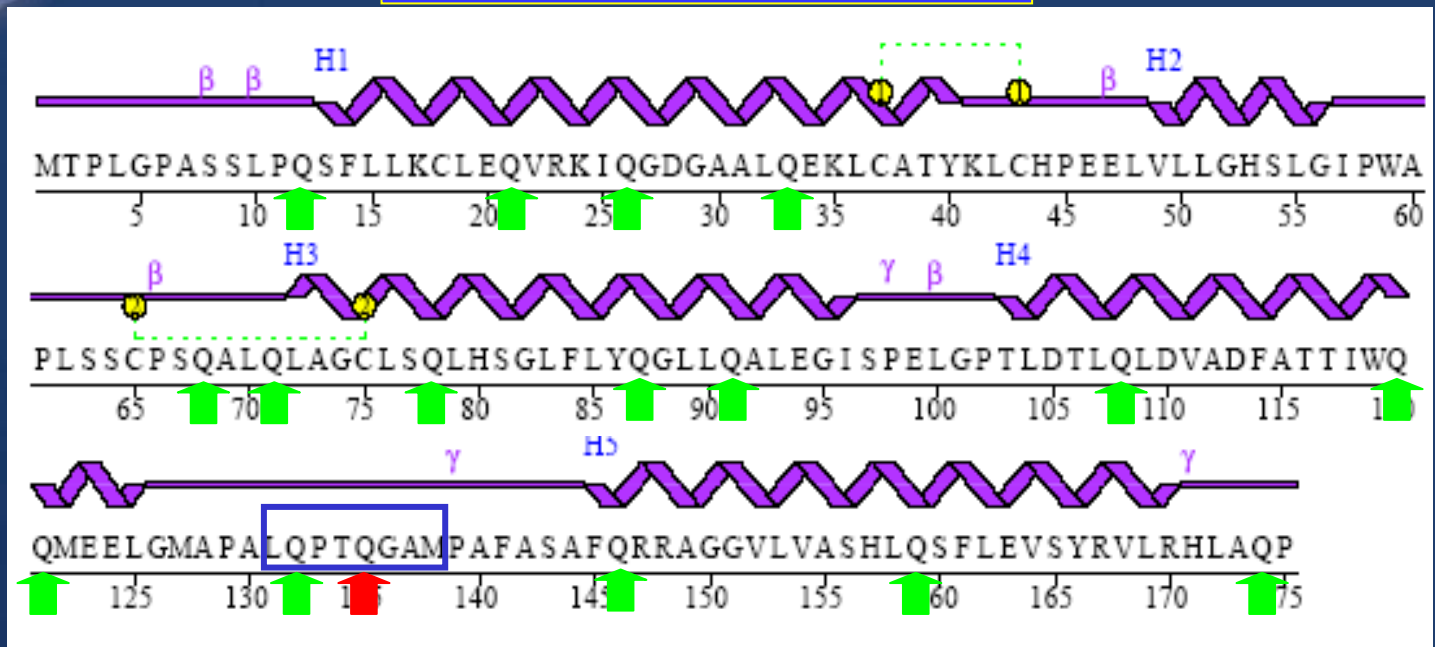
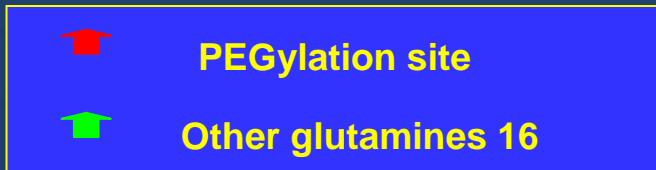
**BK0026**



**Met-G-CSF**



# BK0026: enzymatic PEGylation reaction





## Reaction and purification scheme

### Pegylation with TGase



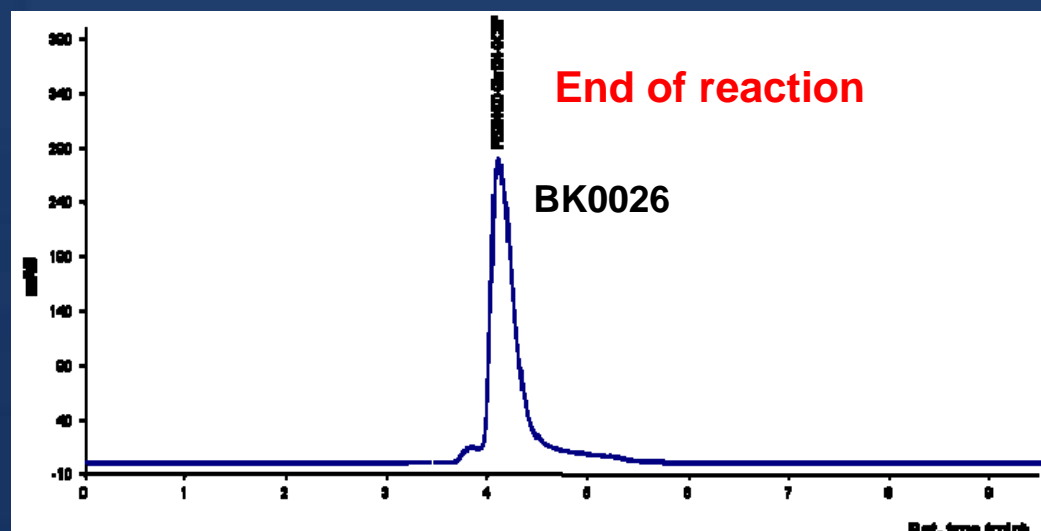
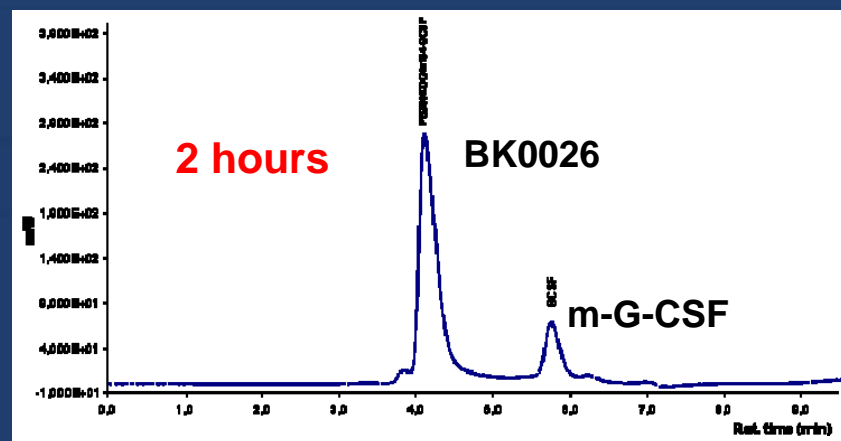
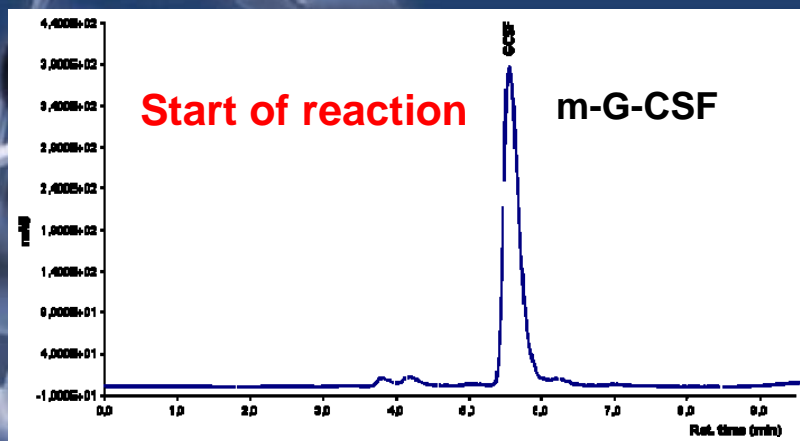
**Ion Exchange Chromatography**  
separation from byproducts

**Gel filtration chromatography**  
Reduction of the solution ionic strength

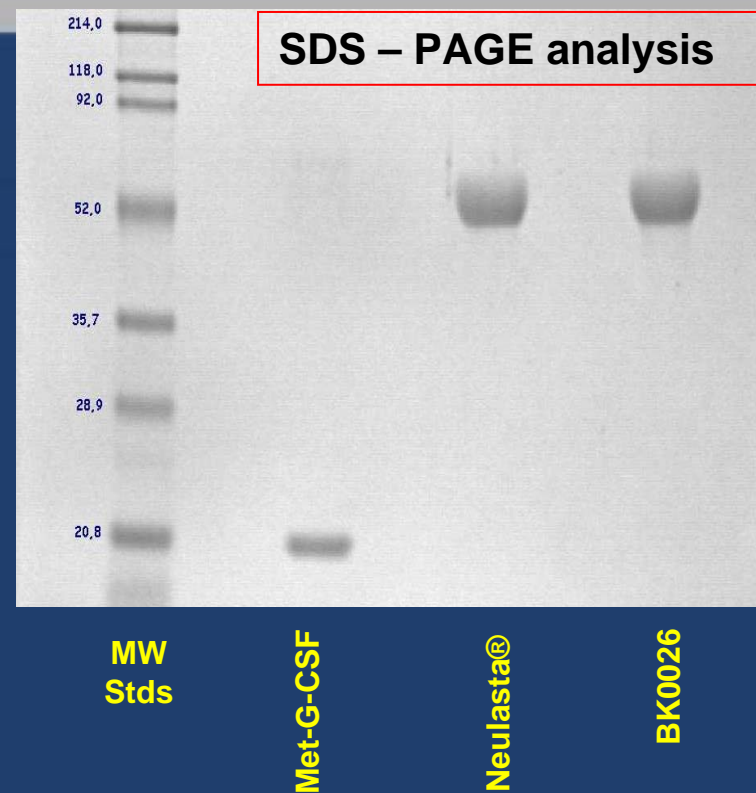
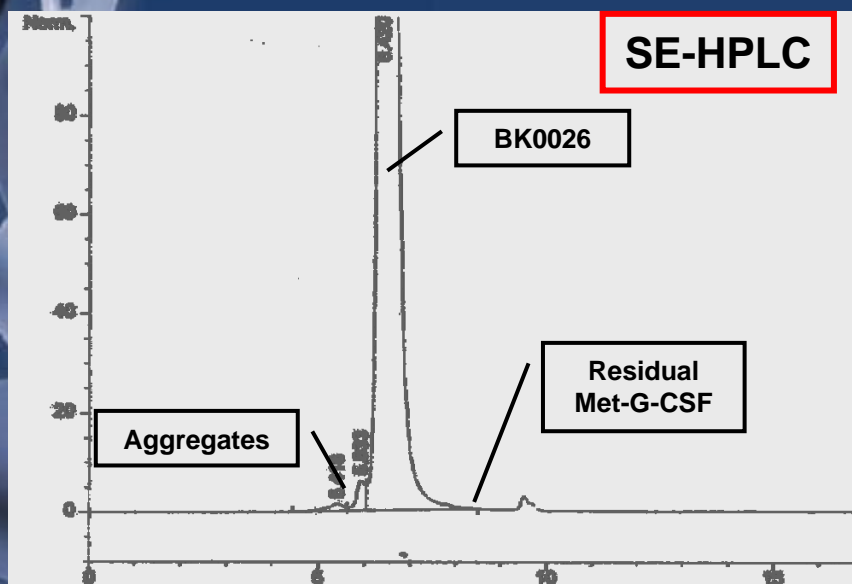
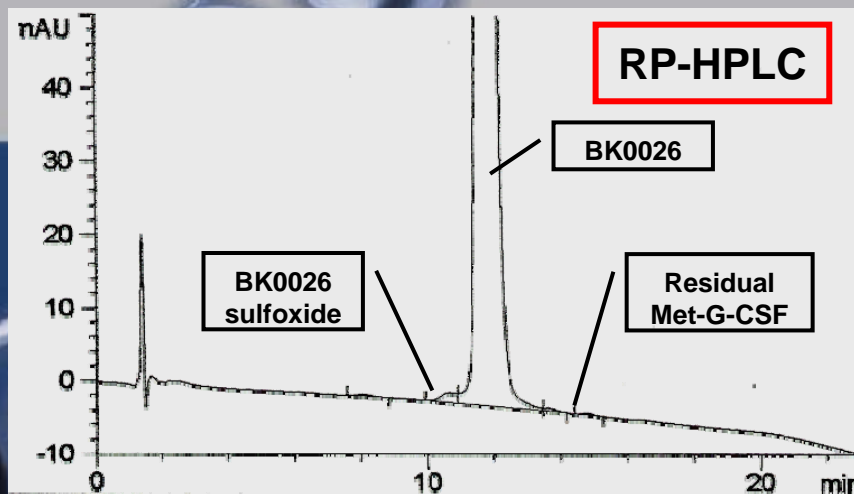
**Ultrafiltration**  
BK0026 concentration

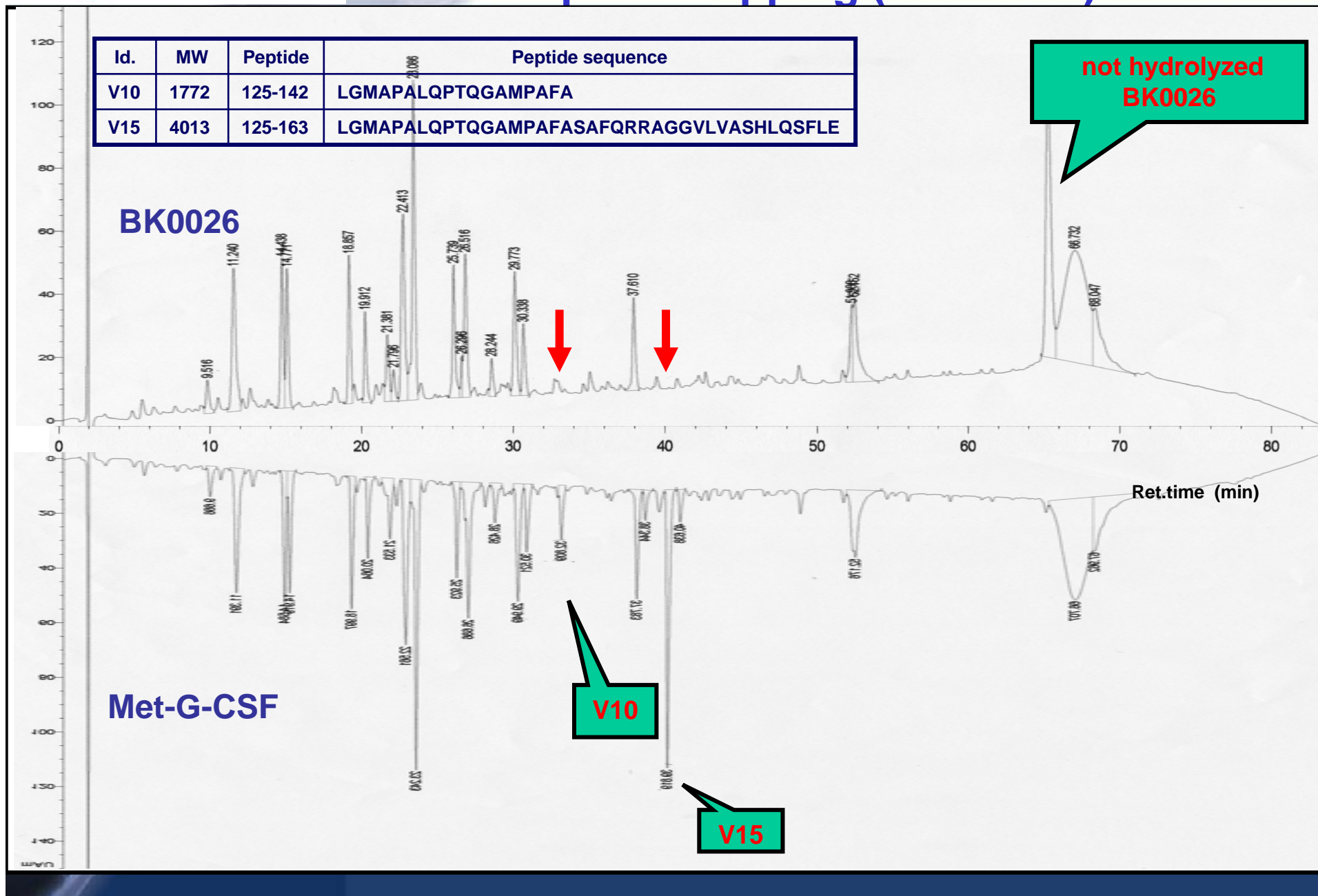
	Analytical Technique	Parameter
<b>Structural characterization</b>	Peptide mapping MS SDS-PAGE SE-HPLC	Primary structure Molecular weight Molecular hindrance Molecular hindrance
<b>Identity</b>	SDS-PAGE/WB RP-HPLC IE-HPLC IEF	Immunological binding Hydrophobicity Charge Charge
<b>Purity (Product related impurities)</b>	RP-HPLC SE-HPLC IE-HPLC IEF	Related proteins and residual G-CSF
<b>(Process related impurities)</b>	SDS-PAGE SDS-PAGE/WB	Residual PEG Residual Tgase
<b>Potency</b>	NFS60 cell proliferation	Biological activity
<b>Quantity</b>	SE-HPLC	Amount

Met-G-CSF and 20kDa PEG reaction mixture in the presence of M-TGase (SE-HPLC analysis)



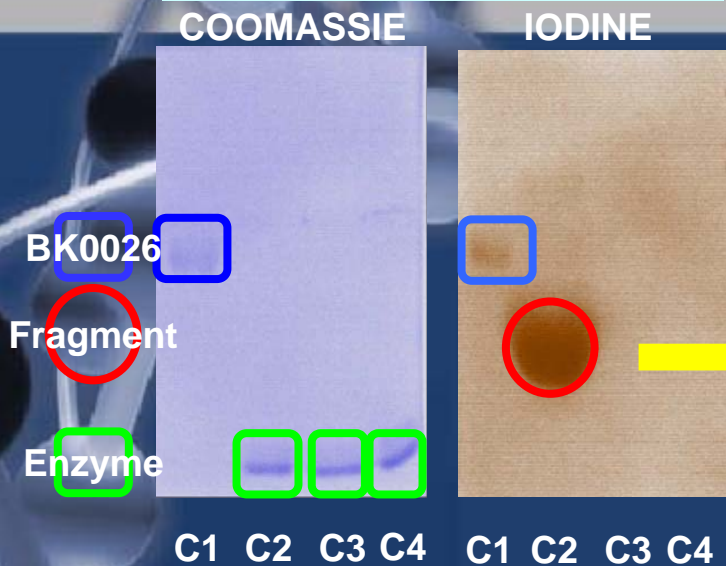
## Identification and purity evaluation



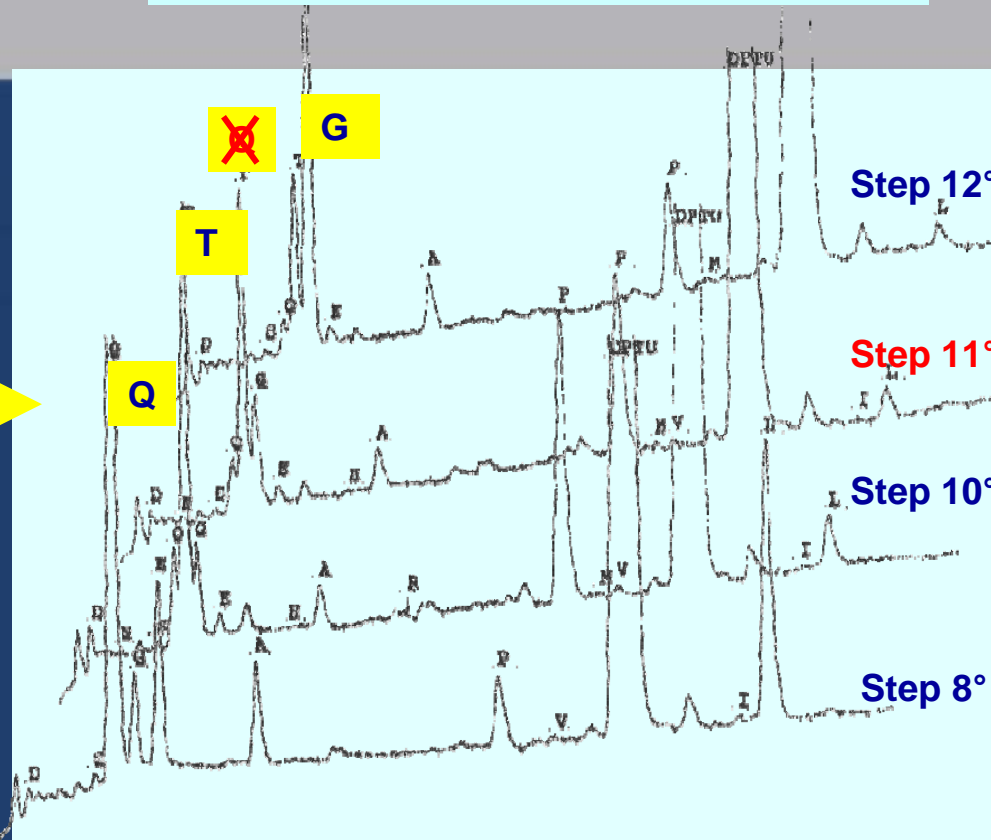


BK0026 hydrolyzed with  
Glu-C enzyme (V8)

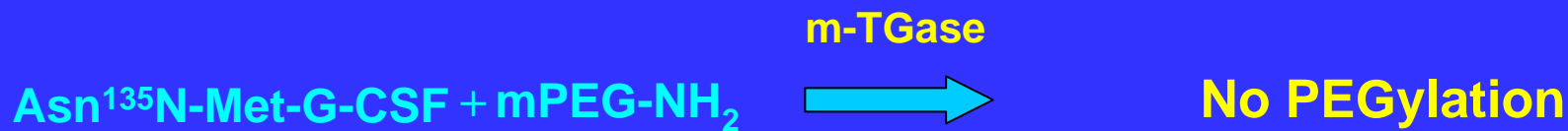
Edman degradation of PEG-peptides



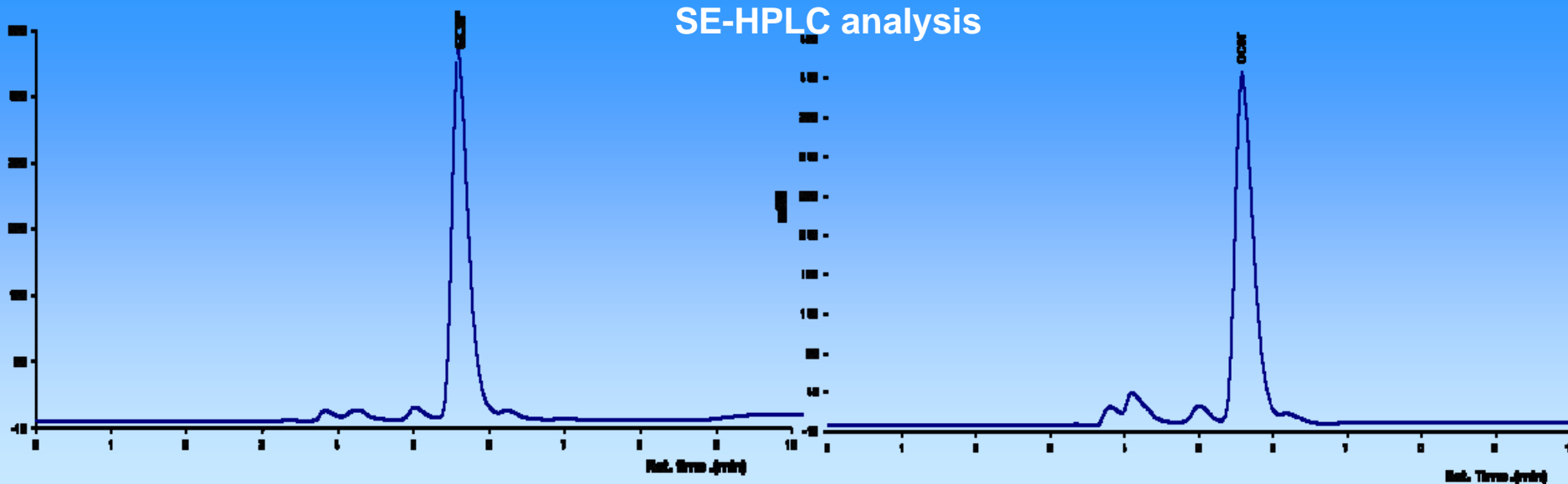
C1 = BK0026 not-hydrolyzed  
C2 = BK0026 Glu-C hydrolyzed  
C3 = self-digested Glu-C  
C4 = Glu-C (V8 endoprotease)



Id.	Peptide	Peptide sequence
V10	125-142	LGMAPALQPTQGAMPFAFA
V15	125-163	LGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLE



SE-HPLC analysis



Start

After 2 hours

### PHARMACOLOGY

#### *In vitro*

- biological potency in NFS-60 cell line
- receptor binding assay
- STAT 3 phosphorylation

#### *In vivo*

- pharmacodynamic and pharmacokinetic profiles after repeated administration at 3 dose levels in neutropenic and non-neutropenic rats

### TOXICOLOGY

- single dose toxicity in rats and mice
- repeated-dose (100, 300 and 1000 µg/kg/ every 3 days) toxicity study in rats (4 weeks)
- local tolerability study in rabbits

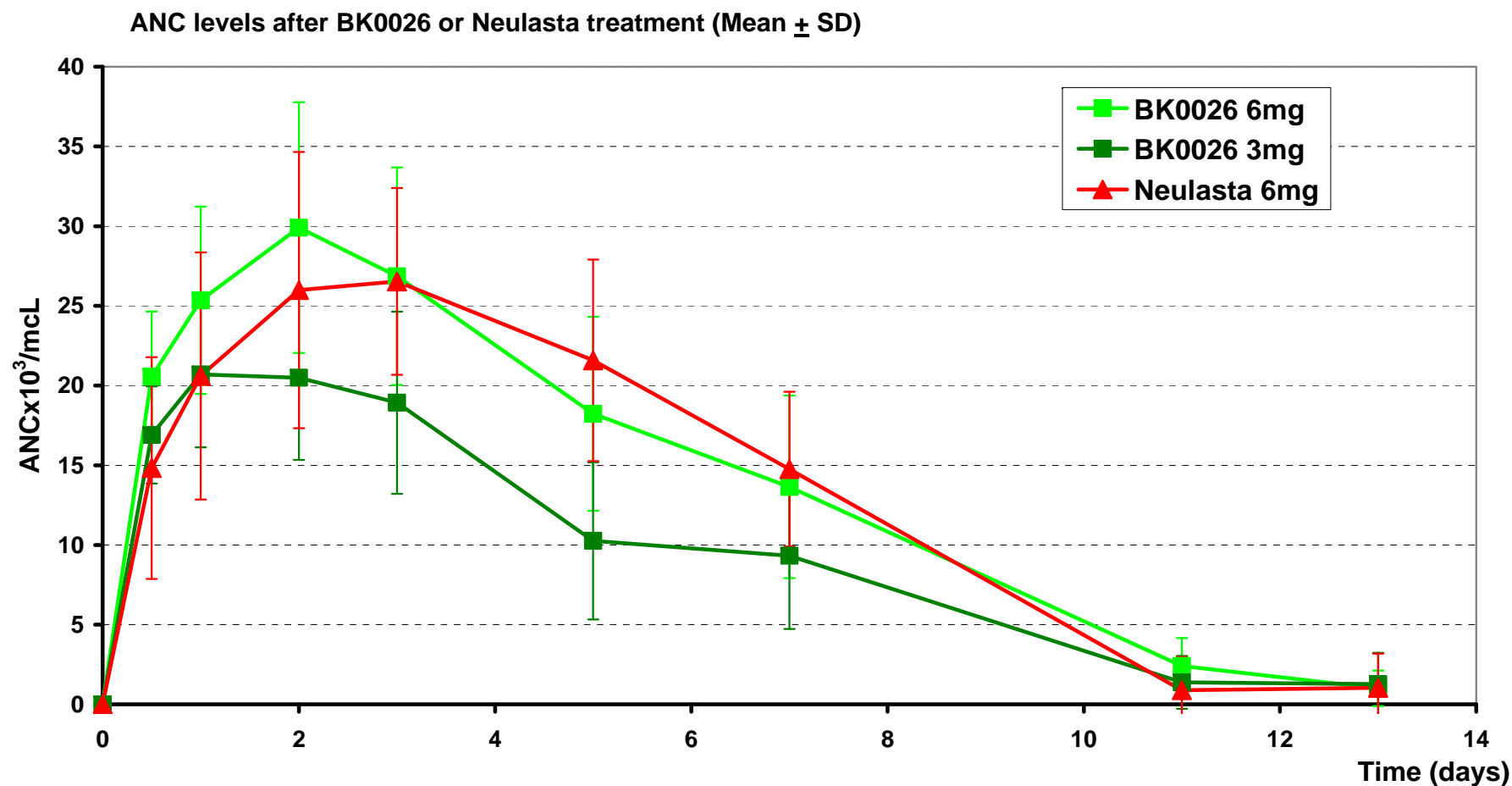
**The biological activity of BK0026 was not significantly different to Neulasta® even if some differences were present.**

Comparative evaluation of pharmacokinetics and pharmacodynamics in male healthy volunteers after a single dose subcutaneous administration of BK0026 (3 and 6 mg) vs. Neulasta® (6mg).

<b><u>Study site</u></b>	<b>Cross Research SA, Arzo, Switzerland</b>
<b><u>Study objective</u></b>	<b>To investigate PK profiles and PD effects of BK0026 at two dosages in comparison to Neulasta® after single dose administration</b>
<b>Sample size</b>	<b>24 randomized subjects</b>
<b>Dosage 1</b>	<b>8 subjects receiving BK0026 at 3mg 1st Cohort (2 subjects) +2ndCohort (2 subjects)+3rd Cohort (4 subjects)</b>
<b>Dosage 2</b>	<b>16 subjects treated in cross over with BK0026 and Neulasta® at 6mg 4<sup>th</sup> Cohort (4 subjects) + 5<sup>th</sup> Cohort (12 subjects)</b>
<b><u>End points</u></b>	
<b>Primary</b>	<b>- PD effects in term of ANC</b>
<b>Secondary</b>	<b>- PD effects in terms of CD34+, PK and safety evaluation</b>

## Preliminary pharmacodynamic (ANC) evaluation

Eight healthy volunteers have been treated with a single 3mg dose of BK0026 (dark green line) and sixteen healthy volunteers have been treated in cross-over with a single 6mg dose of BK0026 (light green line) and Neulasta (red line).



### Preliminary safety evaluation


**The following adverse events (AEs) were reported.**

**Backache or headache of mild intensity were the most frequent AEs: these AEs were present in both the BK0026 and Neulasta groups.**

**Generally the AEs occurred on Day 1 and terminated on the same day or by Day 2 or 3.**

**All these episodes were judged as being related to the study drug and are also reported as side effects in the Neulasta leaflet.**

**No clinically relevant changes in laboratory safety parameters and no clinically relevant modifications of systolic, diastolic blood pressure, heart rate, respiratory rate or body temperature were reported.**

- 
- **Fast recovery of neutropenia.**
  - **Biological half-life more consistent with different chemotherapy protocols.**
  - **New compound: new clinical application could be found.**
  - **New clinical trials for defining the product profile with different chemotherapeutic protocols.**
  - **Competitive cost reduction.**

**EMA Scientific Advice will clarify the following aspects:**

➤ **Additional preclinical studies**

- **toxicological, pharmacodynamic and pharmacokinetic.**

➤ **Additional Phase I studies:**

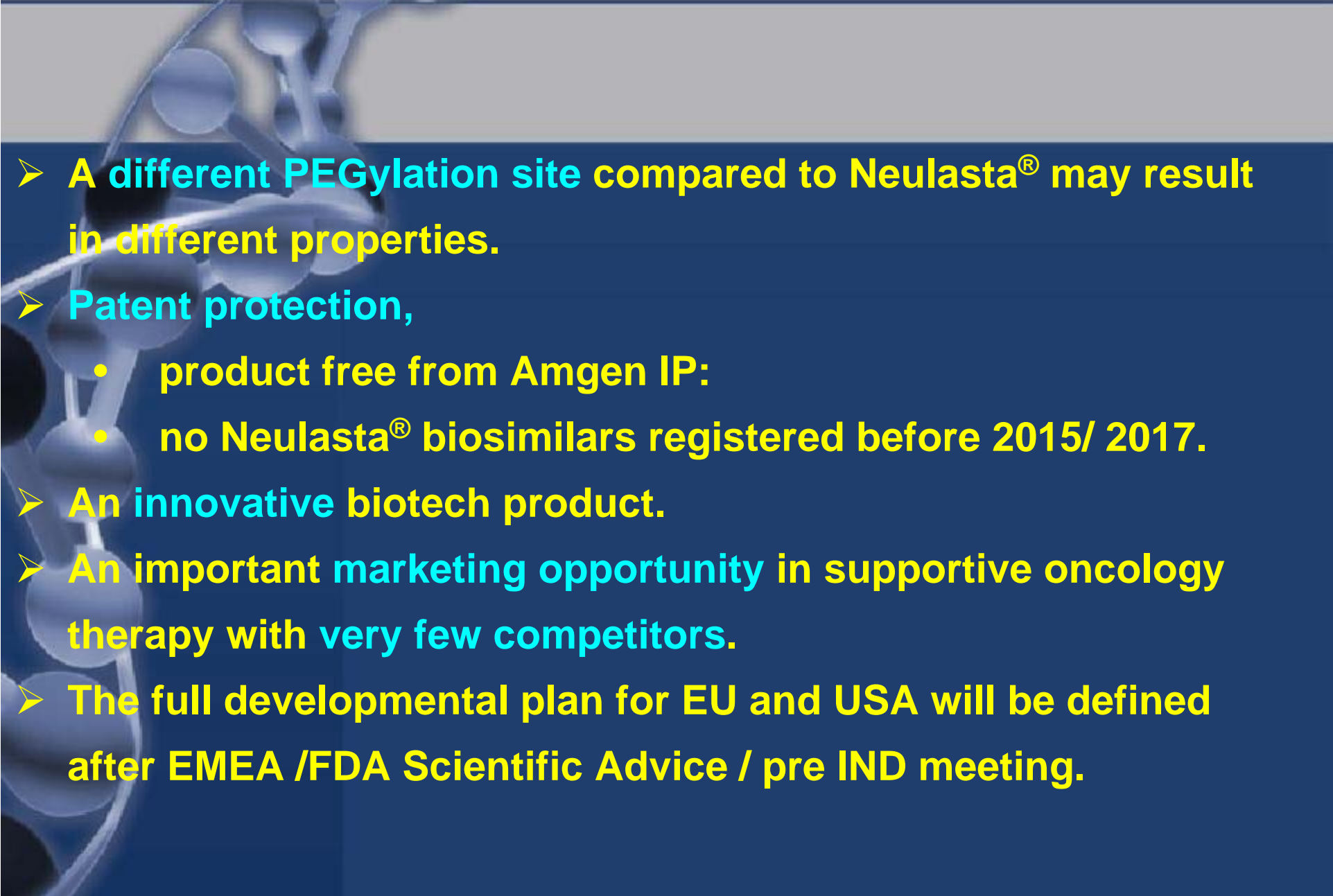
- **Additional subjects, full dose response, pharmacokinetic after iv administration.**

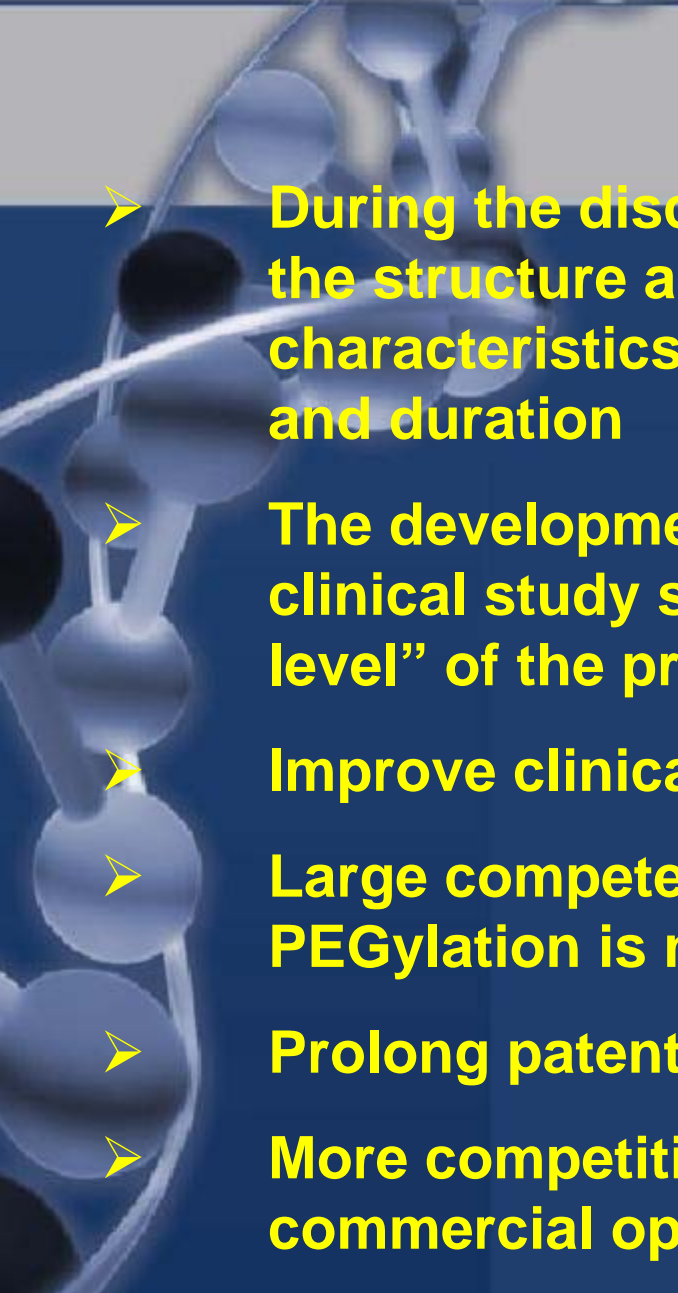
➤ **Phase II study**


➤ **Pivotal Phase III study**

➤ **Paediatric investigations (*EC Regulation No 1901/2006*)**


**All preclinical and clinical studies will be comparative.**

- 
- **A different PEGylation site compared to Neulasta<sup>®</sup> may result in different properties.**
  - **Patent protection,**
    - **product free from Amgen IP:**
    - **no Neulasta<sup>®</sup> biosimilars registered before 2015/ 2017.**
  - **An innovative biotech product.**
  - **An important marketing opportunity in supportive oncology therapy with very few competitors.**
  - **The full developmental plan for EU and USA will be defined after EMEA /FDA Scientific Advice / pre IND meeting.**

- 
- **During the discovery phase it is important to define the structure and PEGylation site, the PEG characteristics, the stability, the biological efficacy and duration**
  - **The developmental plan for both preclinical and clinical study should be related to the “ innovation level” of the product**
  - **Improve clinical efficacy and safety**
  - **Large competence in protein biochemistry and PEGylation is needed**
  - **Prolong patent protection**
  - **More competitive compound in the market with a commercial opportunity**

- 
- **New PEGylated proteins – no**
  - **PEGylated proteins containing an already approved protein moiety - no**
  - **Pegylated proteins differing from the already approved ones by the PEG (size and or type) or by the PEGylation site - probably no**
  - **PEGylated proteins identical to that already approved (PEG and PEGylation site) - yes**

**Development plan have to be defined case by case through Scientific Advice**

- 
- **Demonstration of non inferiority (or better) clinical efficacy**
  - **Safety and tolerability equal or better**
  - **Possibly reduced immunogenicity**
  - **More convenience in drug administration**
  - **Price ?**



Thank you