Appendix E

UNIVERSITÀ DEGLI STUDI DI MILANO

Milan, 21/07/06

Dear Dr. Uzan,

enclosed you will find the report on the inhibition of contact system by eno and the eno-related compounds

Sincerely

Prof. Luigi Bergamschini

UNIVERSITÀ DEGLI STUDI DI MILANO

IN VITRO INHIBITION OF Aβ1-42–DEPENDENT ACTIVATION OF CONTACT/KININ SYSTEM BY ENOXAPARIN AND ENO-DERIVED COMPOUNDS

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Introduction

The contact-kinin system is a proteolytic system comprising four proteins that are present in biological fluid in form of zymogens, namely Hageman factor (f.XII), factor XI (f.XI), pre-kallikrein (PK) and high molecular weight kininogen (HK). Zymogens upon interaction with negatively charged surfaces become activated, triggering a series of enzymatic events that are important in the initiation and amplification of local inflammatory reactions. During activation, F.XII is converted to the catabolic peptides f.XIIf and f.XIIa which activate the classical pathway of Complement system by acting at the site of C1r and C1s (Fig.1).

Alternatively, increased fibrinolytic activity may result in activation of early Complement factors since plasmin can activate C1s in vitro and in vivo(Naff, et al., 1968, Ratnoff, et al., 1967, Ratnoff, et al., 1969). The possible involvement of the kinin system and fibrinolysis in the inflammatory phenomena in the brain of patients with Alzheiemer's disease (AD) is supported by several lines of evidences: i) Hageman factor is associated with amyloid-ß (Aß) deposits(Yasuhara, et al., 1994), ii) HK is massively cleaved in cerebrospinal fluid of AD patients (Bergamaschini, L., et al., 2001), iii) plasminogen, plasminogen activator and inhibitors are produced in brain (Hua, et al., 2002, Kranenburg, et al., 2005). The mechanism of Aß-dependent Contact system activation (Figure 1) involves the interaction of Aß with f.XII and/or kallikrein since f.XII deficiency and kallikrein inhibition abrogate the ability to cleave HK. Furthermore, in vitro, Aß enhances the t-PA dependent conversion of plasminogen to plasmin, as effectively as fibrin(ogen) fragments.

The negatively charged residues, and their distribution within the region 1–11 of Aß (Figure 2) seems to play a key role in the activation of this system as suggested by the finding that there was not a significant difference in the HK cleaving activities of the full length peptides (1– 42 or 1–40) and the truncated fragments 1–28 and 1–11, whereas the fragments Aß12-28, Aß (Gln^{11})1–16 and Aß 40–11 had no activating ability (Bergamaschini L, et al.).

Contact/Kinin system and Allzheiemer's disease

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Several lines of evidence support the hypothesis that contact system could be involved in AD pathogenesis (Joseph, et al., 1999, Bergamaschini, et al., 1998, Niwano, et al., 1995, Yasuhara, et al., 1994). However, whether or not the functional disturbances of neurons or glial cells in AD brain might be, at least in part, attributable to the generation of bradykinin, as a consequence of contact-kinin system activation , remains to be ascertained. In any case, activation of factor XII and pre-kallikrein results in generation of enzymes that react with C1-INH to form f.XIIa-C1-INH and kallikrein-C1-INH complexes. C1-INH is also the major inhibitor of activated complement C1. In situ hybridization revealed that in brain areas with neuritic plaques and activated glial cells, only neurons express C1-INH mRNA and not the other cells such as astrocytes that usually produce them. Thus, a defective synthesis combined to an increased rate of consumption of C1-INH may lead to a functional deficiency of C1-INH. This local C1-INH functional deficiency may facilitate the activation of contact/kinin system, as well as of complement, at the site of affected areas of AD brain (Sarvari, et al., 2003).

Amyloid plaques and neurofibrillar tangles (NFTs), the neuropathological hallmarks of AD, may not be the direct cause of the symptoms, however there is a large body of evidence that Aß could be implicated in pathogenesis of AD because of its cytotoxicity and the ability to trigger a robust, local inflammatory reaction (Cotman, et al., 1996, Veerhuis, et al., 2005).

Possible beneficial actions of Heparin in AD

Evidence that Aß accumulation is likely to contribute to AD pathology, provides the rationale for a therapy based on altering brain Aß accumulation and/or reducing its cytotoxic and proinflammatory actions . In vitro charged residues within the region 1-11 are critical for Aß proinflammatory activity (Bergamaschini, et al 2001, Velazquez, et al., 1997, Webster, et al., 1997) thus inhibition of this activity by pharmacological targeting of this region, might be useful to slow progression of neurodegeneration in AD brain. Candidates for such a therapy could be heparin? (Leveugle, et al., 1998, Leveugle, et al., 1994, Tyrrell, et al., 1999, Watson, et al., 1997) that binds

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the 12-17 region of Aß. While most of the studies on the heparin structure and function have concentrated on understanding interactions responsible for inhibition of blood coagulation, the observation that heparin-containing tissues are those in direct contact with the external environment suggests a role in host defence.

In recent years researches, both *in vitro* and *in vivo*, highlighted a broad range of anti-inflammatory applications of heparin and of the low molecular weight heparin enoxaparin.

It has been shown that heparins induce *in vitro*:

1) the inhibition of Aβ production and deposition (Chen, et al., 1995 Akiyama, 1997, Akiyama, et al., 1992, Arai, T., et al., 2006, Evin, et al., 2003, Gardella, et al., 1990, Gardella, et al., 1992, Grammas, et al., 2004, Hino, et al., 2001);

2) the removal of metal cations, Zn and Cu, reducing aggregation of Aβ and the release of reactive oxygen species from cells (Curtain, et al., 2003, Engelberg, 2004, Esler, et al., 1996, Jiang, et al., 1995, Karr, et al., 2004, Kowalik-Jankowska, et al., 2001, Kozin, et al., 2001, Liu, et al., 1999, Van Nostrand, 1995);

3) the inhibition of arginine and lysine effects throughout the binding to positively charged Apolipoprotein E ($ApoEe_4$) residues, thus minimizing $ApoEe_4$ effects and providing a beneficial action in AD pathogenesis (Bazin, et al., 2002, Dong, et al., 2001, Libeu, et al., 2001, Sadowski, et al., 2004, Saito, et al., 2003, Shuvaev, et al., 2000).

4) the inhibition of Aß-dependent activation of complement and conatact/kinin system, , and cytotoxic effect of Aß (Velazquez, et al., 1997; Bergamaschini, et al., 2002; Bergamaschini, et al. 2004)

In vivo chronic treatment of APP23 transgenic mice (over-expressing human APP_{751}) with a prophylactic dose of enoxaparin results in a significant reduction in number and area occupied by amyloid deposits in the neocortex, compared to saline-treated APP23 mice(Bergamaschini, et al..

2004). Furthermore, enoxaparin treatment before and during reperfusion reduces myocardial infarct size and tissue inflammatory reaction (Libersan, et al. Cardiovasc Res. 1998)

Aim of the Study

Enoxaparin, as well as all LMWHs in clinical use, is a mixture of several saccharides of different length, and possibly structure that could have an important role in the biological activity of these drugs, as suggested by the demonstration that LMWHs have different antithrombotic activity.
In order to determine the relationship between structure and anti-inflammatory activity, 15 compounds (enoxaparin-derived, and heparin related) have been evaluated for their ability to inhibit the Aβ-dependent activation of the contact system.

Material and methods

Aβ peptide preparation. Aβ(1-42) (Bachem AG, Bubendorf) was prepared by dissolving lyophilized Aβ in high quality H_2Od at a concentration of 1mg/ml and immediately diluted to 100μg/ml with PBS (pH 7.4).

Eno-derived compounds preparation. According to your suggestion, compounds were prepared by dissolving lyophilised compounds in high quality H_2Od at a concentration of 84mg/ml, and stored at 4°C.

Mixture of plasma /Aß(1-42)/ Compound preparation. In preliminary experiments normal Na-citrated plasma was draw from healthy volunteers, and the level of HK cleavage was evaluated trough SDS-PAGE and immunoblotting. Only plasmas with no, or very low, evidence of HK self-activation (absence of the III band on immunoblotting) were used for the activation/inhibition experiments. Suitable plasma samples were stored in small aliquots at -70°C until used; plasma samples were snap thawed at 37°C immediately before their used, and used only once.
Freshly solubilized Aβ (50μl) was mixed with a fixed volume of Eno or Eno-derived compounds, to obtain the established weight/weight ratios. Within 5 min, Aß-Compound mixtures were added to an equal plasma samples and incubated at 37°C for 30 min. The reaction was quenched by adding PBS containing soybean trypsin inhibitor (100μg/ml), EDTA (10mM), Polybrene (0.05%vol./wt).

Contact system activation. Contact system activation was assessed by measuring the degree of cleavage of HK, a suitable marker of contact system activation using densitometric analysis of immunostained blotting membranes after SDS-PAGE. This method simultaneously evaluates the native protein and its activation fragment. Plasma samples were loaded on 8% acrylamide gel and separated by SDS under non-reducing conditions. Proteins were transferred to polyvinylidene-difluoride membranes by electroblotting and HK bands were visualized with polyclonal goat anti-light chain HK and biotin conjugated rabbit anti goat IgG and an avidin-alkaline phosphatase substrate. The blotting membranes were analyzed with a high-performance scanner and Image Master software. The level of HK activation was expressed as the percentage of total protein (band II + III versus bands I + II + III). The apparent masses of native HK and its activation fragments were Mw 130,000 (band I), 110,000 (band II) and 98,000 (band III). Inter-assay variation was 15%, intra-assay variation was 7%.

Results

Experiments were performed comparing compound according to your suggestions as follow:

Compound 1 vs Compound 5 (Figure 3)

Compound 1 inhibits more efficiently than Compound 5:

- Compound 1 looses activity between 1:25 and 1:10 ratios;
- Compound 5 has no or very low inhibitory activity at 1:50 ratio and show no inhibition at lower weight ratios.

Compound 2 vs Compound 6 (Figure 4)

Both the Compounds have inhibitory activity, particularly Compound 2 being more active than Compound 6:

- Compound 2 looses its activity between 1:10 and 1:1 ratios;
- Compound 6 does not inhibit at all at 1:10 weight ratio.

Compound 3 vs Compound 7 (Figure 5)

Compound 7 show a better inhibitory action than Compound 3:

- Compound 7 is able to inhibit HK activation up to 1:10, but does not inhibits at 1:1 ratio;
- Compound 3 stars to loose activity at weight ratio 1:25, has a very low activity at 1:10 and has no activity at 1:1 ratio.

Compound 4 vs Compound 8 (Figure 6)

Compound 4 inhibits more efficiently than Compound 8:

- Compound 4 has a strong inhibitory activity up to 1:1 ratio;
- Compound 8 has no inhibitory activity below 1:25 ratio.

Compound 9 vs Compound 10 vs Compound 11 *(Figure 7)*

Compound 11 inhibits better than Compound 9 and 10. The Compound 9 seems to be better than Compound 10.

- Compound 11 shows a powerful inhibitory action up to 1:1 ratio;
- Compound 9 starts to loose activity at 1:1 ratio;
- Compound 10 shows no inhibition at 1:1 ratio.

Compound 12 vs Compound 13 vs Compound 14 (Figure 8)

Compound 12 seems to be more efficiently than Compound 13 and 14 that exhibit a similar inhibitory effect:

- Compound 12 looses its inhibitory activity at weight ratio 1:10;
- Compound 13 and Compound 14 show a similar activity up to 1:50 ratio and loose activity starting from 1:25.

Compound 15 vs Enoxaparin (Figure 9)

Enoxaparin has a powerful inhibitory effect than Compound 15:

- Enoxaparin retains activity up to weight ratio 1:1;
- Compound 15 does not show any activity at ratio 1:10.

Conclusion

In previous studies it has been shown that contact/kinin system may be involved in the inflammatory reactions in AD brain, and that its activation occurs trough the interaction of HK to the negatively charged residues of Aß. Enoxaparin, that binds to Aß1-42, was able to reduce the toxic effect of amyloid both in vitro and in vivo. Since enoxaparin contains a mixture of saccharides of different length and possibly structure, to determine the relationship between structure and activity , in this study we compare 15 compounds isolated from enoxaparin or enoxaparin like for their ability it inhibit Aß-dependent activation of contact/kinin system.

Any of the compounds, per se, increased the activity of Aß1-42 on the HK activation

We found a great variability in the inhibitory activity: compounds 5 and 8 exhibiting the lowest activity, whereas compounds 2, 4 and 11 retained inhibitory activity even at 1:1 ratio.

These differences in the inhibitory activity could indicate a specific contribution of each saccharide to the overall anti-inflammatory activity of enoxaparin, and suggest a possible structure/activity relationship.

Abbreviation:

LMWH= low molecular weight heparin

ENO = enoxaparin

PK= prekallikrein

f..XII= Hageman factor

HK= high molecular weight kininogen

C1-INH = inhibitor of activated complement C1

Aß = Alzheimer amyloid-ß protein

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Milan, 20.07.2006

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FIGURES

Figure 1. Schematic rapresentation of the interaction between complement and contact-kinin cascades. In AD brain, Aß-dependent activation of contact-kinin system can lead to activation of Complement classical pathway through generation of the activation products of Hageman factor (F.XII). The two systems are passible of reciprocal activation and share C1-INH as a major inhibitor, thus whatever system is activated first, chronic activation induced by Aß can lead to local deficiency of C1-INH.

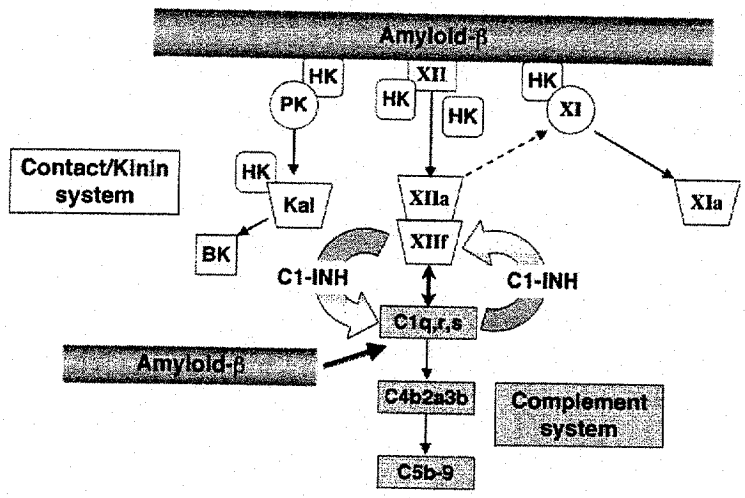


Figure 2. Sequence of Alzheimer Aß1-42. Residues D^7 and E^{11} are critical for complement activation; positively charged residues (H, K) within the 12-18 region are involved in the binding to heparin. Negatively charged residues are underlined.

1 11 15

D-A-E-F-R-H-D-S-G-Y- V-H-H-

-K-L- -F-F-A-E-D-V-G-S-N-

16 22

-G-A-I-I-G-L-M-V-G-G-V-V-I-

29 42

Legend to Figures 3-9

A. A representative Western-blotting analysis of the cleavage of high molecular weight kininogen (HK). The Aß/compound ratios are reported at the bottom of each lane.

Inter-assay variation (compound *vs* compound): 15%

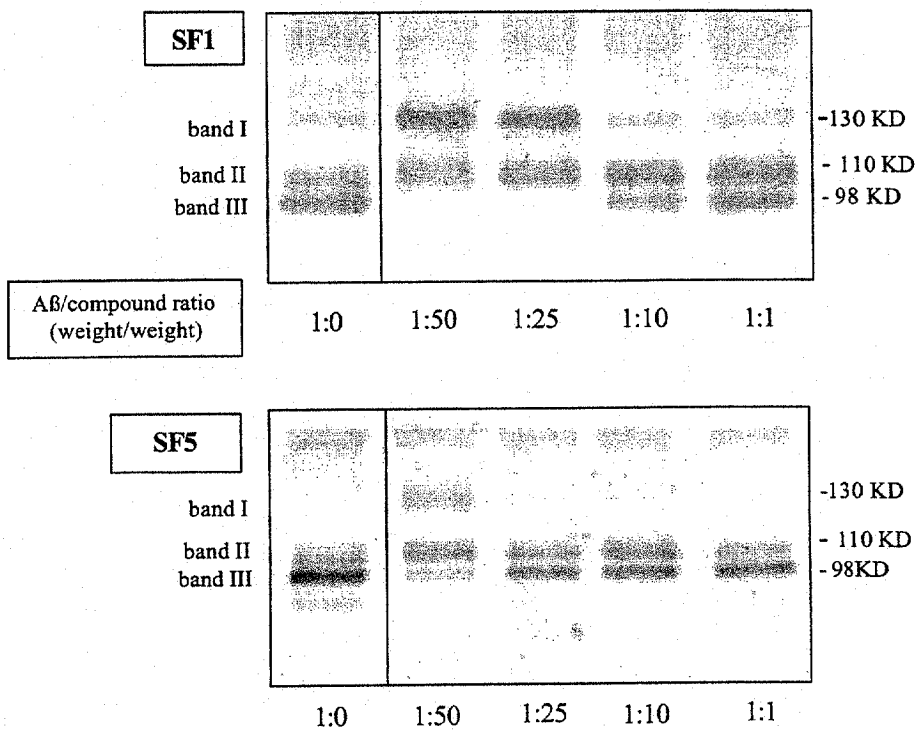
Intra-assay variation (Aß/compound ratio *vs* Aß/compound ratio): 7%.

B. Columns are the level of HK activation at the different Aß/compound ratio, expressed as percentage of the activation induced by Aß alone (1:0 ratio).

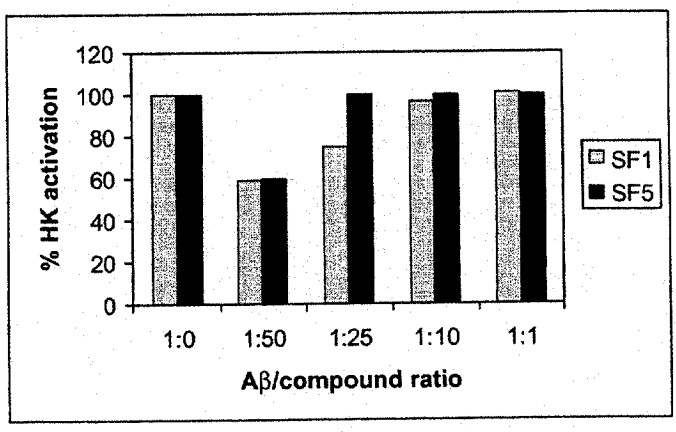
Percentage of Aß induced activation (1:0 ratio) = 100%.

Compound 1 versus Compound 5 (Figure 3)

A



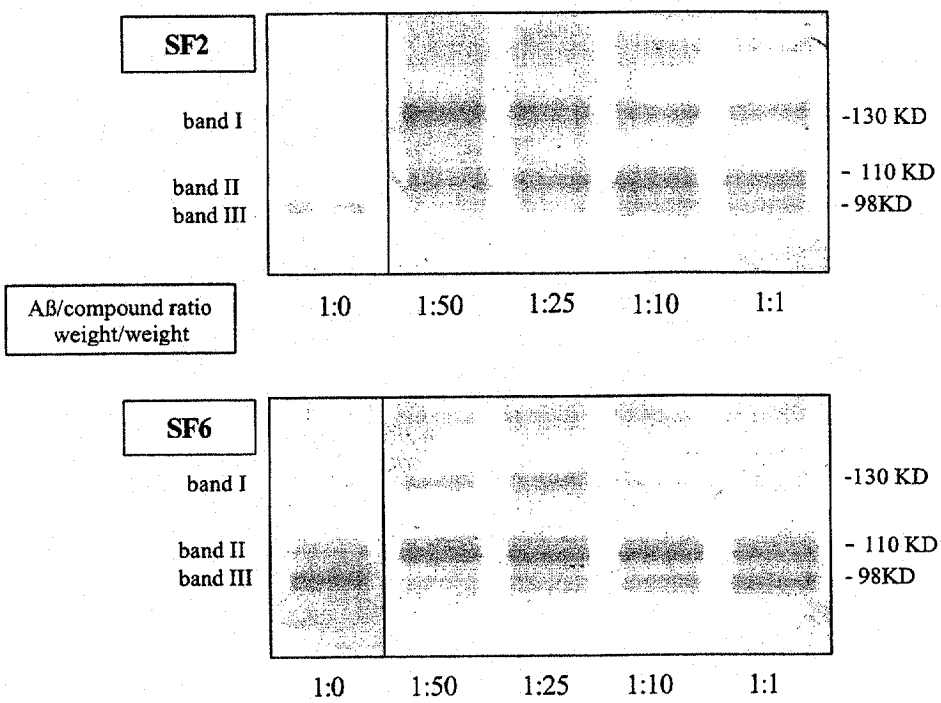
B



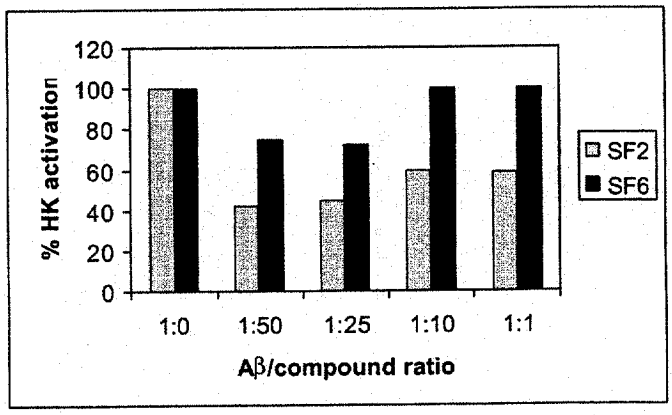
	1:0	1:50	1:25	1:10	1:1
SF1 % activation	100%	59%	75%	97%	101%
SF5 % activation	100%	60%	100%	100%	100%

Compound 2 versus Compound 6 (Figure 4)

A



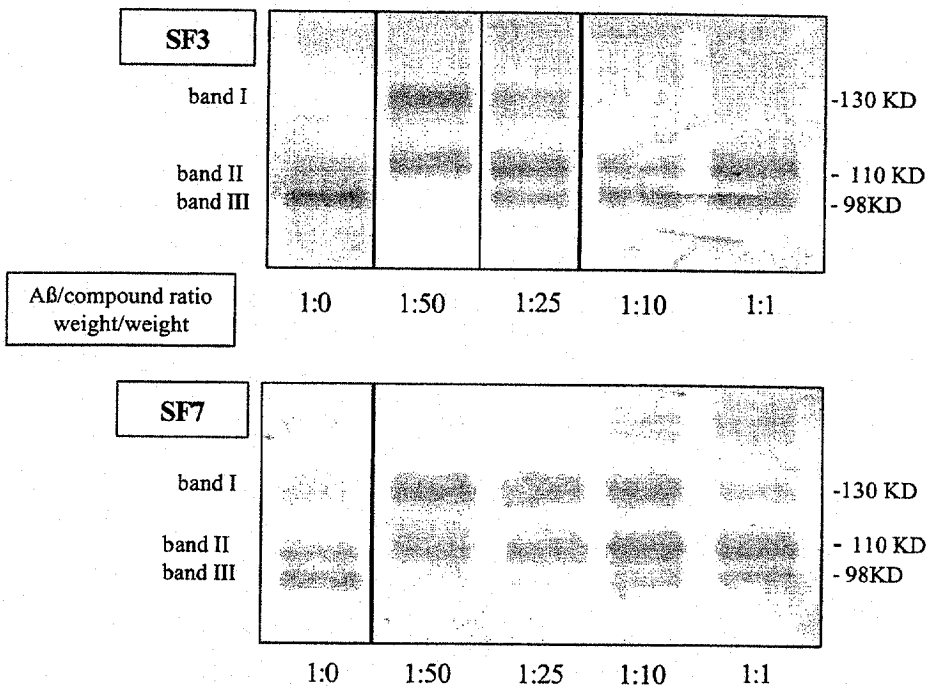
B



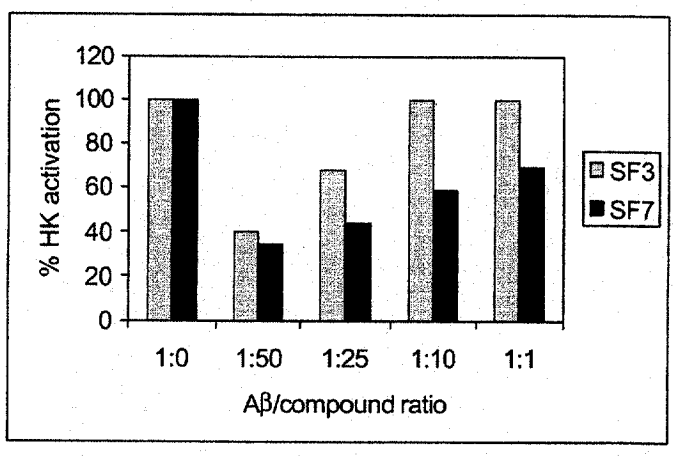
	1:0	1:50	1:25	1:10	1:1
SF2 % activation	100%	42%	45%	60%	59%
SF6 % activation	100%	75%	73%	100%	100%

Compound 3 versus Compound 7 (Figure 5)

A



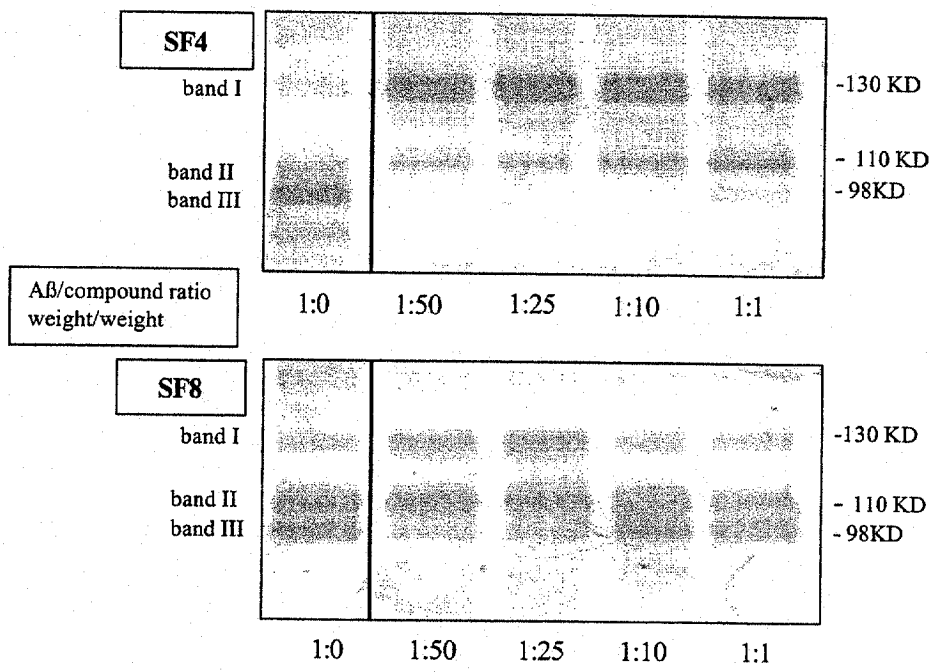
B



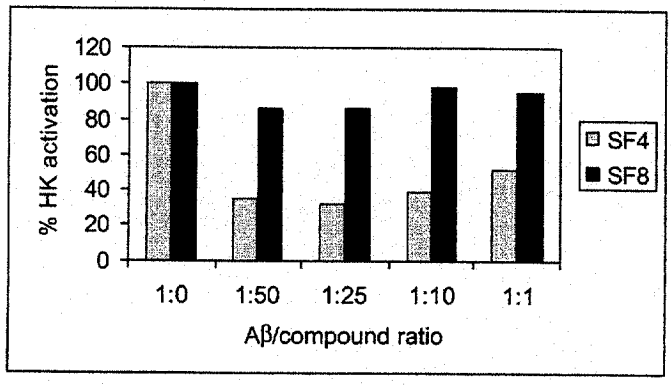
	1:0	1:50	1:25	1:10	1:1
SF3 % activation	100%	40%	68%	100%	100%
SF7 % activation	100%	35%	44%	59%	70%

Compound 4 versus Compound 8 (Figure 6)

A



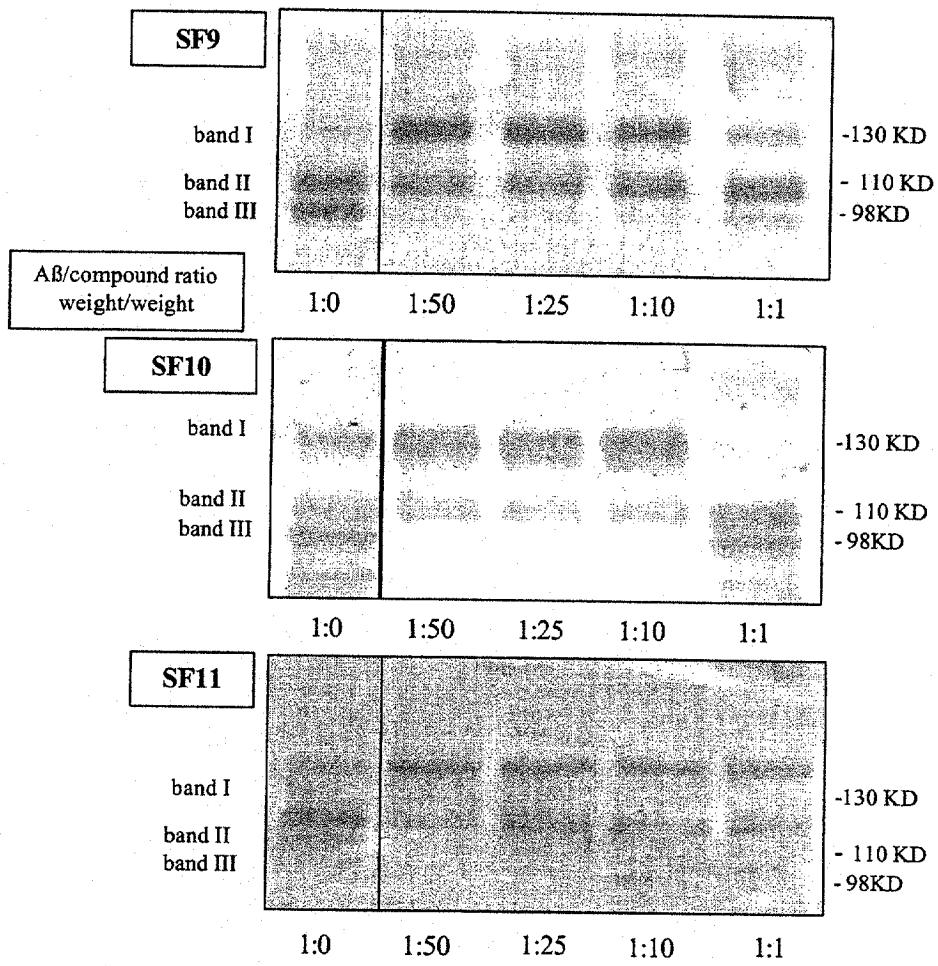
B



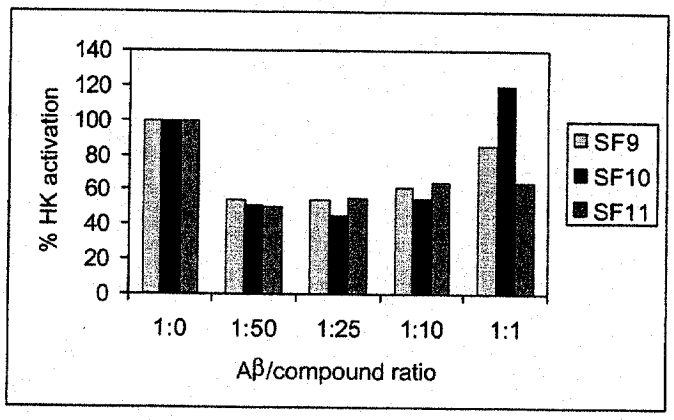
	1:0	1:50	1:25	1:10	1:1
SF4 % activation	100%	35%	32%	39%	52%
SF8 % activation	100%	86%	86%	98%	96%

Compound 9 versus Compound 10 versus Compound 11 (Figure 7)

A



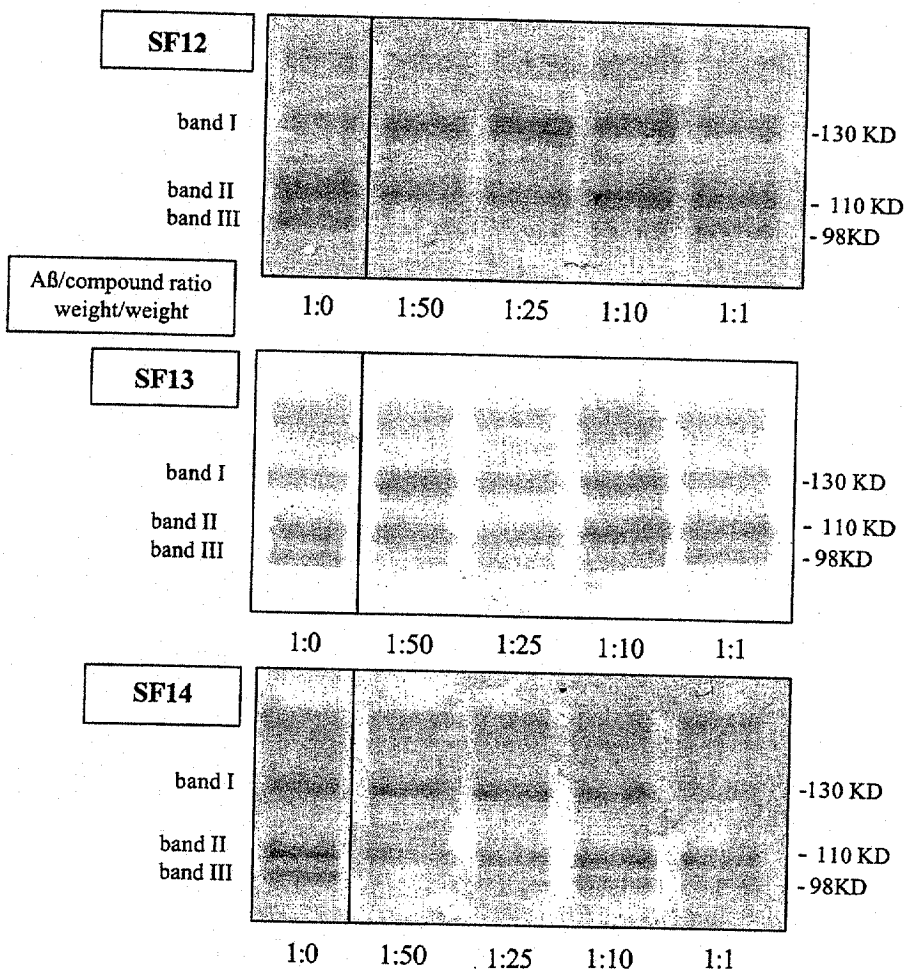
B



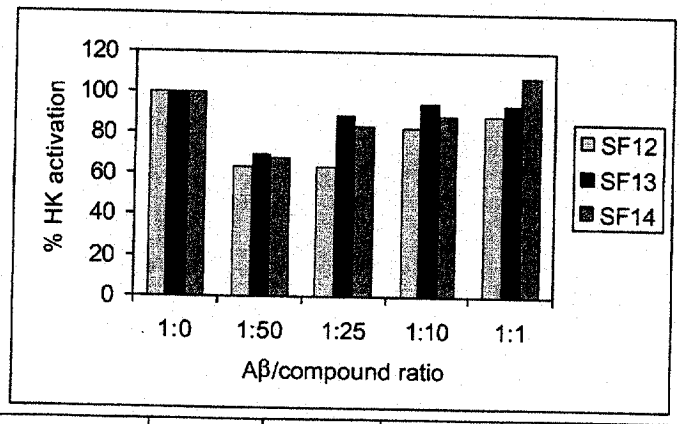
	1:0	1:50	1:25	1:10	1:1
SF9 % activation	100%	54%	54%	61%	86%
SF10 % activation	100%	51%	45%	55%	120%
SF11 % activation	100%	50%	56%	65%	65%

Compound 12 versus Compound 13 versus Compound 14 (Figure 8)

A



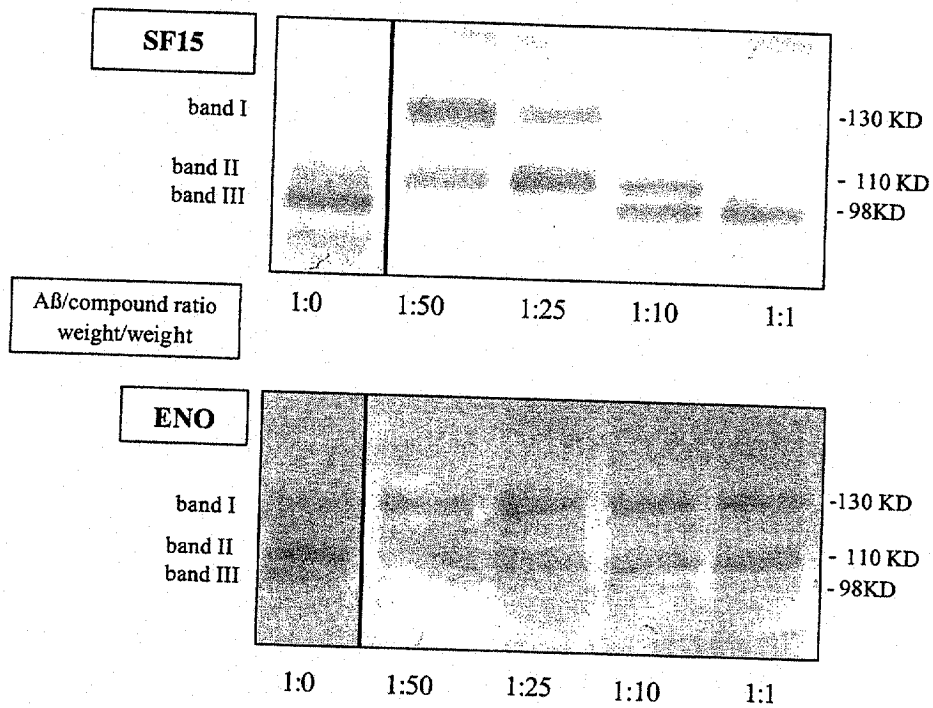
B

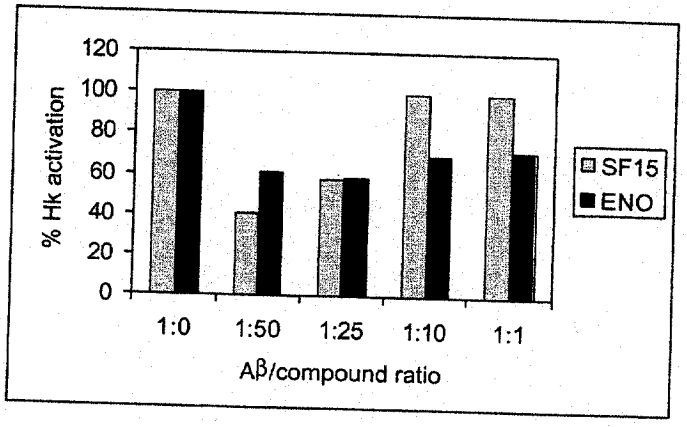


	1:0	1:50	1:25	1:10	1:1
SF12 % activation	100%	64%	64%	83%	89%
SF13 % activation	100%	70%	89%	95%	94%
SF14 % activation	1005	68%	84%	89%	108%

Compound 15 versus enoxaparin (Figure 9)

A





	1:0	1:50	1:25	1:10	1:1
SF15 % activation	100%	41%	58%	100%	100%
ENO % activation	100%	61%	59%	70%	73%