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(54)名稱

能夠取代 FVIII(A)之高效 ISVD 化合物

(57)摘要

本發明係提供能夠結合凝血因子 IX(a)及因子 X(a)的 ISVD 多肽衍生物，其具有很高的效力並提供足夠長的半衰期，可以有效地進行皮下及口服投予。本文所揭露之 ISVD 多肽衍生物因而適於藉由不同投予途徑（包括皮下及口服投予）來治療 A 型血友病、具有抑制子之 A 型血友病、及後天性 A 型血友病。

The present invention provides ISVD polypeptide derivatives capable of binding coagulation Factor IX (a) and Factor X(a) which are highly potent and provide a sufficiently long half-life such to allow for effective subcutaneous- as well as peroral administration. The ISVD polypeptides derivatives disclosed herein are thus suitable for treatment of haemophilia A, haemophilia A with inhibitors and acquired haemophilia A by various routes of administration including subcutaneous and peroral administration.

指定代表圖：

【圖1a】



公告本

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【發明摘要】

【中文發明名稱】 能夠取代FVIII(A)之高效ISVD化合物

【英文發明名稱】 HIGHLY POTENT ISVD COMPOUNDS CAPABLE OF SUBSTITUTING FOR FVIII(A)

【中文】

本發明係提供能夠結合凝血因子IX(a)及因子X(a)的ISVD多肽衍生物，其具有很高的效力並提供足夠長的半衰期，可以有效地進行皮下及口服投予。本文所揭露之ISVD多肽衍生物因而適於藉由不同投予途徑（包括皮下及口服投予）來治療A型血友病、具有抑制子之A型血友病、及後天性A型血友病。

【英文】

The present invention provides ISVD polypeptide derivatives capable of binding coagulation Factor IX(a) and Factor X(a) which are highly potent and provide a sufficiently long half-life such to allow for effective subcutaneous- as well as peroral administration. The ISVD polypeptides derivatives disclosed herein are thus suitable for treatment of haemophilia A, haemophilia A with inhibitors and acquired haemophilia A by various routes of administration including subcutaneous and peroral administration.

【指定代表圖】 圖1a

【代表圖之符號簡單說明】

無

【特徵化學式】

無

【發明說明書】

【中文發明名稱】 能夠取代FVIII(A)之高效ISVD化合物

【英文發明名稱】 HIGHLY POTENT ISVD COMPOUNDS CAPABLE OF SUBSTITUTING FOR FVIII(A)

【技術領域】

【0001】 本發明係有關能夠結合凝血因子IX(a)及因子X(a)的化合物以及其在治療凝血病（諸如不同形式的血友病，包括A型血友病）的用途。

序列表之引用併入

【0002】 本申請案以電子形式提交序列表。序列表之整體內容在此以引用方式併入。

【先前技術】

【0003】 在患有凝血功能障礙(coagulopathy)的患者中，諸如在患有A型血友病(HA)及B型血友病(HB)的人類中，凝血級聯之各種步驟由於諸如功能性凝血因子之不存在或存在不足而變得功能異常。此類凝血級聯之一部分的功能異常導致血液凝血不足及潛在危及生命的出血，或內臟器官(諸如關節)的損傷。

【0004】 凝血因子VIII (FVIII)缺乏(一般稱為A型血友病)為一種全球影響大約42萬人(其中大約10萬5千人目前被診斷出)的先天性出血病症。A型血友病的嚴重程度分為三個等級，由因子FVIII血漿含量1%或更低（「嚴重的」）、2%至5%（「中度的」）、及6%-30%(「輕度的」)所定義(White等人，(2001) Thromb. Haemost. 85:560)，或是根據WFH「血友病管理指南」第二版，Haemophilia；電子版2012年7月6日定義的5%至<40%。出血可以是自發地出現，也可以在外傷後出現。大

約一半的A型血友病患者被歸類為患有嚴重的A型血友病，並從兒童早期開始經歷嚴重的出血，並在以後的生活中經常發生自發性或過度的出血。出血通常發生在關節和肌肉中，如果沒有適當的治療，反復出血會導致不可逆轉的出血性關節病(Manco-Johnson等人，(2007) N. Engl. J. Med. 357: 535-44)。

【0005】 患有A型血友病之患者可接受凝血因子替代療法，諸如外源性FVIII療法。習用治療由替代療法組成，其以出血發作之預防或需求性治療形式提供。直到最近，患有嚴重A型血友病之患者的預防性治療為每週最多3次靜脈注射血漿衍生性FVIII或重組FVIII或其長效型變體。

【0006】 然而，此類患者對於此類外源性因素有發展出中和抗體(所謂的抑制物)的風險，從而使先前的有效療法失效。具有抑制物之A型血友病患者為部分先天性及部分後天性凝血功能障礙的非限制性實例。已發展出FVIII抑制物的患者不能以習用替代療法治療。外源性凝血因子僅可靜脈投予，其對於患者而言相當不便且不適。

【0007】 由FVIII活性減低或缺乏造成的FXa形成不足及凝血酶生成減低係血友病A患者中的出血體質之基本原因。

【0008】 FX進到其酵素活性形式FXa的蛋白分解性轉化可藉由包含有FIXa及其輔因子活化FVIII (FVIIIa)的內在FX活化性複合物達成。輔因子結合增加FIXa之酵素活性達約五個數量級且被認為係透過多個機制造成，如由Scheiflinger等人 (2008) *J Thromb Haemost*, 6:315-322略述的。特別地，已發現FVIIIa會穩定具有增加的針對FX的蛋白分解性活性的FIXa之構形(Kolkman JA, Mertens K (2000) *Biochemistry*, 39:7398-7405, Zögg T, Brandstetter H (2009) *Biol Chem*, 390:391-400)。

【0009】 近幾年，有一種也被稱為ACE910的艾美賽珠單抗(emicizumab) (HEMLIBRA®)已被核准用於具有或沒有針對習用補充治療因子的抑制子之A型

血友病的皮下預防性治療。艾美賽珠單抗為中外製藥公司(Chugai Pharmaceuticals)/羅氏製藥公司(Roche Pharmaceuticals)所開發之用於治療A型血友病的人源化雙特異性全長抗FIX(a)/抗FX(a)單株抗體。艾美賽珠單抗被設計成模擬FVIII輔因子功能(參見Sampei等人(2013) *PLoS One*, 8, e57479及WO2012/067176)。推測每毫升血漿採用30-50 μg的艾美賽珠單抗的治療係對應於每公合血漿至少10至15IU的等效因子VIII活性(Shima等人，N Engl J Med 2016; 374:2044-53)。然而，一些患者已經產生了針對艾美賽珠單抗的抑制子(抗藥物抗體)，而使得採用此化合物的治療無效。

【0010】 除了以艾美賽珠單抗為例生成抑制子外，其他抗體性質對於針對患者實現有效的基於抗體的治療也至關重要。特別是，已經證明了具有高非特異性結合傾向的抗體可能導致臨床安全性問題。在一些報導中，高程度的非特異性結合導致抗體的循環半衰期縮短數倍，並導致針對患者的給藥方案無效且繁瑣(參見Dobson等人，*Nature*，第6卷，文章編號: 38644 (2016)及Avery等人，*MAbs* 2018，第10卷，第2冊, 244–255)。

【0011】 WO2018/141863、WO2019/065795 (US10759870)、WO2020/025672及WO2021/152066公開了抗FIX(a)/抗FX(a)雙特異性全長抗體以及其作為促凝血劑用於皮下投予方式治療血友病的用途。一種這樣的雙特異性抗體被命名為Mim8(參見Østergaard H等人，Blood. 2021;138:1258–68及WO2020/025672)。

【0012】 然而，血友病群體中仍存在許多非常重要之未得到滿足的醫療需求，特別是在患有凝血病的個體中，以及特別是需要減輕治療負擔且能夠替代FVIII(a)用於治療凝血病（諸如A型血友病及相關疾病）的改進化合物。

【發明內容】

【0013】 本發明係提供能夠結合凝血因子IX(a)及凝血因子X(a)的促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物，諸如V_HH多肽衍生物，其具有很高的效力並提供足夠長的半衰期，可以有效地進行皮下投予以及口服投予。因此，在一態樣中本發明係有關一種促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物，其包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合的第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、與一或多個表面暴露殘基接附之一或多個延長部分、一連接ISVD1及ISVD2之可選的連接子(L_{1-2})、以及可選的一或更多個延伸子(E)。

【0014】 在一態樣中本發明係有關一種促凝血ISVD多肽衍生物，其包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合的第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、與一表面暴露殘基接附之至少一延長部分、一連接ISVD1及ISVD2之可選的連接子(L_{1-2})、以及可選的一或更多個延伸子(E)，其中所述第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）中之至少一者的因子IX (SEQ ID NO:1)或其活化型上的表位，且其中所述第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）中之至少一者的因子X (SEQ ID NO:2)上的表位。

【0015】 該ISVD多肽衍生物可例如為V_HH多肽衍生物。

【0016】 本發明之另一態樣係有關於包含有本文所揭露之ISVD多肽衍生物的醫藥組成物。本發明之另一態樣係有關於本文所揭露之ISVD多肽衍生物，以及包含有這類化合物之組成物，在藉由不同投予途徑（包括皮下及口服投予）來治療不同形式的血友病的用途，尤其是A型血友病、具有抑制子之A型血友病、及後天性A型血友病。

【0017】 本發明之又一態樣係有關於其為ISVD多肽衍生物或V_HH多肽衍

生物之一部分的個別組份（中間物）ISVD或V_HH片段，諸如特定的抗FIX(a) V_HH片段或其特定的抗FX(a) V_HH片段。

【0018】 本發明之又一態樣係有關於如本文中所揭露之該等化合物之組份（中間物）的製造，包括用於修飾能夠結合FIX(a)及FX(a)之ISVD多肽衍生物的等電位以改進這類多肽衍生物的口服生物可利用率之方法。

【圖式簡單說明】

【0019】 [圖1a-f]係顯示抗FX(a)/FIX(a) ISVD多肽衍生物之非限制性實例。E：延伸子，PM：延長部分，L_{1-2}：連接ISVD1及ISVD2之連接子。虛線係表示雙硫鍵。

【0020】 [圖2a]係顯示一抗FX(a)/FIX(a) V_HH多肽衍生物之非限制性實例，其包含有單個C18二酸脂肪酸基延長部分通過一延長部分連接子(L_P)(ID: L_P1)與一C端延伸子共軛。虛線係表示雙硫鍵。

【0021】 [圖2b]係顯示一抗FX(a)/FIX(a) V_HH多肽衍生物之非限制性實例，其包含有單個四唑基延長部分通過一延長部分連接子(L_P)(ID: L_P2)與一C端延伸子共軛。虛線係表示雙硫鍵。

【0022】 [圖3a-f]係顯示化合物#20 (a)、化合物#18 (b)、化合物#15 (c)、化合物#13 (d)、化合物#14 (e)及化合物#12 (f)等化合物之詳細圖像說明，其等為抗FX/FIX(a) V_HH多肽衍生物之非限制性實例且所述多肽衍生物包含有一雙C68二酸脂肪酸基延長部分通過一延長部分連接子(L_P)(ID: L_P1)與一C端延伸子(E)共軛。虛線係表示雙硫鍵。該延伸子(E) (SEQ NO:9)係用於這些V_HH多肽衍生物之中。該延伸子分別在位置4及6包含兩個半胱胺酸，作為該等延長部分(PM)的接附點。

在a)中，序列V_HH1.20 - L_{1-2} - V_HH2.20 – E多肽係由SEQ ID NO:634 (化合物

#20)表示。

在b)中，序列$V_HH1.18$ - L_{1-2} - $V_HH2.18$ - E多肽係由SEQ ID NO:632 (化合物#18)表示。

在c)中，序列$V_HH1.15$ - L_{1-2} - $V_HH2.15$ - E多肽係由SEQ ID NO:629 (化合物#15)表示。

在d)中，序列$V_HH1.13$ - L_{1-2} - $V_HH2.13$ - E多肽係由SEQ ID NO:627 (化合物#13)表示。

在e)中，序列$V_HH1.14$ - L_{1-2} - $V_HH2.14$ - E多肽係由SEQ ID NO:628 (化合物#14)表示。

在f)中，序列$V_HH1.20$ - L_{1-2} - $V_HH2.20$ – E多肽係由SEQ ID NO:626 (化合物#12)表示。

【0023】 [圖4]係顯示化合物#6、Mim8及艾美賽珠單抗SIA之滴定曲線（活性為化合物濃度的結果）。

【0024】 [圖5a及5b]係分別顯示抗FIX(a)及抗FX ISVD (V_HH片段)的序列比對，其中CDR序列係以黑體及下劃線標出。

序列簡單說明

【0025】 SEQ ID NO:1係表示人類凝血因子IX之胺基酸序列。

SEQ ID NO:2係表示人類凝血因子X之胺基酸序列。

SEQ ID NO:3-13及690係表示延伸子(E)的胺基酸序列。

SEQ ID NO:14-26及691係表示L_{1-2}及L_P連接子的胺基酸序列。

SEQ ID NO:27-614係表示V_HH片段及其互補性決定區的胺基酸序列。

SEQ ID NO:615-691、734及735係表示包括有任何L_{1-2}連接子及/或延伸子之V_HH多肽的胺基酸序列。

SEQ ID NO:692-733係表示如本文實例4中所揭露之肽片段的序列。

SEQ ID NO:736-739係表示潛在的延長子之胺基酸序列。

【實施方式】

【0026】 本發明係提供能夠結合因子FIX(a)及FX(a)的ISVD多肽衍生物，其具有很高的效力並提供足夠長的半衰期，可以有效地進行皮下投予以及口服投予。本文所揭露之ISVD多肽衍生物因而適於藉由不同投予途徑（包括皮下及口服投予）來治療不同形式的血友病，諸如A型血友病、具有抑制子之A型血友病、及後天性A型血友病。

【0027】 尤其，本發明係有關雙特異性經延長ISVD多肽，諸如稱為ISVD多肽衍生物（或V_HH多肽衍生物）之V_HH多肽，其可結合共軛FIXa及共軛FX，導致形成活化的共軛FX (FXa)，其方式是模仿凝血因子VIIIa (FVIIIa)的輔助因子活性。本文所揭露之V_HH多肽衍生物顯示出高度的活體內效價，且其(例如)比雙特異性抗體艾美賽珠單抗序列一致類似物(SIA)高幾個數量級。該V_HH多肽衍生物在動物模型（諸如大鼠、狗、豬）中亦經由導入的延長子（脂肪酸共軛及白蛋白結合肽融合）而顯示出半衰期延長。未延長的V_HH多肽在狗及豬中則顯示出非常快速的清除率。再者，該V_HH多肽衍生物係經工程改造以經由對表面暴露的殘基進行降低pI的胺基酸取代，以及使用賦形劑N-(8-(2-羥基苯甲醯基)胺基)辛酸鈉(SNAC)及(例如)菸鹼醯胺(NAM)的製劑，使口服投予後的生物可利用率具有臨床意義。因此，當口服投予時，所調製之高效V_HH多肽衍生物顯示出大鼠及狗的動物模型中之生物可利用率水平據臨床相關性。迄今為止，諸如ISVD多肽衍生物之分子量超過10 kDa的治療性多肽（諸如V_HH多肽衍生物）被認為不適合口服投予。然而，本文所揭露之V_HH多肽衍生物係適用於新的凝血病（諸如具有或不具有抑制子之A型血友病）口服治療方式。

【0028】 本發明因此提供能夠結合凝血因子IX(a)及凝血因子X(a)的促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物（諸如V_HH多肽衍生物），其具有很高的效力並提供足夠長的半衰期，可以有效地進行皮下投予以及口服投予。因此，在一態樣中本發明係有關一種促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物，其包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合的第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、與一或多個表面暴露殘基接附之一或多個延長部分、一連接ISVD1及ISVD2之可選的連接子(L_{1-2})、以及可選的一或更多個延伸子(E)。圖1a-f係顯示抗FX/FIX(a) ISVD多肽衍生物的非限制性實例。

【0029】 在一態樣中本發明係有關一種促凝血ISVD多肽衍生物，其包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合的第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、與一表面暴露殘基接附之至少一延長部分、一連接ISVD1及ISVD2之可選的連接子(L_{1-2})、以及可選的一或更多個延伸子(E)，其中所述第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）中之至少一者的因子IX (SEQ ID NO:1)或其活化型上的表位，且其中所述第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）中之至少一者的因子X (SEQ ID NO:2)上的表位。

【0030】 本發明之另一態樣係有關促凝血V_HH多肽衍生物，包含

一第一V_HH (V_HH1)，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二V_HH (V_HH2)，其能夠與因子X (SEQ ID NO:2)或其活化型結合，

至少一延長部分，其係與一表面暴露殘基接附，

一可選的連接子(L_{1-2})，其連接V_HH1及V_HH2，及

可選的一或更多個延伸子(E)，

其中V_HH1包含以下序列

V_HH-2.20 (SEQ ID NO:171)，

V_HH-2.18 (SEQ ID NO:155)，

V_HH-2.15 (SEQ ID NO:131)，

V_HH-2.13 (SEQ ID NO:115)，

V_HH-2.14 (SEQ ID NO:123)，

V_HH-2.12 (SEQ ID NO:107)，或

V_HH-2.2 (SEQ ID NO:35)，

且其中V_HH2包含以下序列

V_HH-1.20 (SEQ ID NO:167)，

V_HH-1.18 (SEQ ID NO:151)，

V_HH-1.15 (SEQ ID NO:127)，

V_HH-1.13 (SEQ ID NO:111)，

V_HH-1.14 (SEQ ID NO:119)，

V_HH-1.12 (SEQ ID NO:103)，

V_HH-1.3 (SEQ ID NO:31)，或

V_HH-1.4 (SEQ ID NO:39)。

【0031】 在一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.20 (SEQ ID NO:167)。

【0032】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.18 (SEQ ID NO:151)。

【0033】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.15 (SEQ ID NO:127)。

【0034】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID

NO:171)且V_HH2係包含序列V_HH-1.13 (SEQ ID NO:111)。

【0035】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.12 (SEQ ID NO:103)。

【0036】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.3 (SEQ ID NO:31)。

【0037】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.4 (SEQ ID NO:39)。

【0038】 在一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.20 (SEQ ID NO:167)。

【0039】 在另一這類實施例中，V_HH1係包含序列V_HH-2.18 (SEQ ID NO:155)且V_HH2係包含序列V_HH-1.18 (SEQ ID NO:151)。

【0040】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.15 (SEQ ID NO:127)。

【0041】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.13 (SEQ ID NO:111)。

【0042】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.12 (SEQ ID NO:103)。

【0043】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.3 (SEQ ID NO:31)。

【0044】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.4 (SEQ ID NO:39)。

【0045】 在另一這類實施例中，V_HH1係包含序列V_HH-2.15 (SEQ ID NO:131)且V_HH2係包含序列V_HH-1.18 (SEQ ID NO:151)。

【0046】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID

NO:171)且V_HH2係包含序列V_HH-1.15 (SEQ ID NO:127)。

【0047】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.13 (SEQ ID NO:111)。

【0048】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.12 (SEQ ID NO:103)。

【0049】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.3 (SEQ ID NO:31)。

【0050】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.4 (SEQ ID NO:39)。

【0051】 在另一這類實施例中，V_HH1係包含序列V_HH-2.13 (SEQ ID NO:115)且V_HH2係包含序列V_HH-1.18 (SEQ ID NO:151)。

【0052】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.15 (SEQ ID NO:127)。

【0053】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.13 (SEQ ID NO:111)。

【0054】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.12 (SEQ ID NO:103)。

【0055】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.3 (SEQ ID NO:31)。

【0056】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.4 (SEQ ID NO:39)。

【0057】 在另一這類實施例中，V_HH1係包含序列V_HH-2.14 (SEQ ID NO:123)且V_HH2係包含序列V_HH-1.18 (SEQ ID NO:151)。

【0058】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID

NO:171)且V_HH2係包含序列V_HH-1.15 (SEQ ID NO:127)。

【0059】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.13 (SEQ ID NO:111)。

【0060】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.12 (SEQ ID NO:103)。

【0061】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.3 (SEQ ID NO:31)。

【0062】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.4 (SEQ ID NO:39)。

【0063】 在另一這類實施例中，V_HH1係包含序列V_HH-2.12 (SEQ ID NO:107)且V_HH2係包含序列V_HH-1.18 (SEQ ID NO:151)。

【0064】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.15 (SEQ ID NO:127)。

【0065】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.13 (SEQ ID NO:111)。

【0066】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.12 (SEQ ID NO:103)。

【0067】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.3 (SEQ ID NO:31)。

【0068】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.4 (SEQ ID NO:39)。

【0069】 在另一這類實施例中，V_HH1係包含序列V_HH-2.2 (SEQ ID NO:135)且V_HH2係包含序列V_HH-1.18 (SEQ ID NO:151)。

【0070】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID

NO:171)且V_HH2係包含序列V_HH-1.15 (SEQ ID NO:127)。

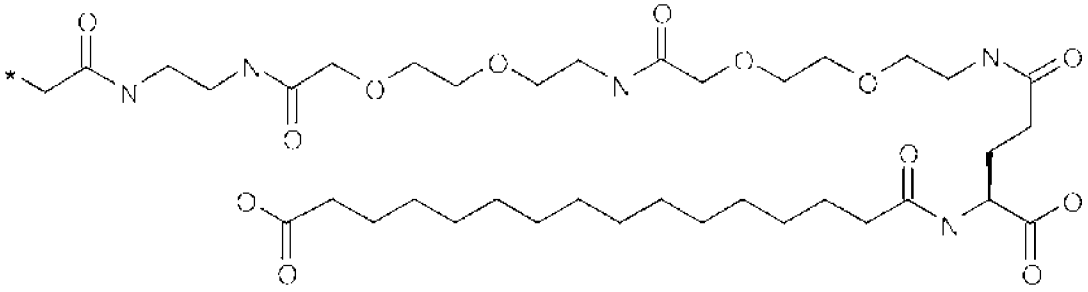
【0071】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.13 (SEQ ID NO:111)。

【0072】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.12 (SEQ ID NO:103)。

【0073】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.3 (SEQ ID NO:31)。

【0074】 在另一這類實施例中，V_HH1係包含序列V_HH-2.20 (SEQ ID NO:171)且V_HH2係包含序列V_HH-1.4 (SEQ ID NO:39)。

【0075】 在一些實施例中該延長部分包含以下結構：



其中*表示該ISVD多肽接附的位點。

【0076】 該ISVD多肽衍生物可例如為V_HH多肽衍生物。

【0077】 在另一態樣中，本發明係有關於包含有本文所揭露之ISVD多肽衍生物的醫藥組成物。本發明之另一態樣係有關於本文所揭露之ISVD多肽衍生物，以及包含有這類化合物之組成物，在藉由不同投予途徑（包括(但不限於)皮下及口服投予）來治療不同形式的血友病的用途，尤其是A型血友病、具有抑制子之A型血友病、及後天性A型血友病。

【0078】 本發明之又一態樣係有關於其為ISVD多肽衍生物或V_HH多肽衍生物之一部分的個別組份（中間物）ISVD或V_HH片段，諸如特定的抗FIX(a) V_HH

片段或其特定的抗FX(a) V_HH片段。

【0079】 本發明之又一態樣係有關於如本文中所揭露之該等化合物之組份（中間物）的製造，包括用於修飾能夠結合FIX(a)及FX(a)之ISVD多肽衍生物的等電位以改進這類多肽衍生物的口服生物可利用率之方法。

定義

【0080】 為了可更易於理解本發明，某些術語的定義如下。

【0081】 希臘字母可由其符號或相應的書面名稱呈現，例如：α= 阿爾法(alpha)；β= 貝他(beta)；ε= 伊普西隆(epsilon)；γ= 伽馬(gamma)；ω= 亞米茄(omega)；等。再者，希臘字母μ可由「u」表示，例如,μl=ul，或μM=uM。

【0082】 化學式中的星號(*)係表示接附點。

【0083】 術語「一」或「一個」旨在意指「一或多個」。當對步驟或元件進行敘述時，術語「包含(comprise)」及其變化型（諸如「包含了」及「包含有」）係意欲表示視情況且不排除添加其他步驟或元件。

【0084】 本文中所用的術語「約」意指大約、粗略或大概。當術語「約」與數值範圍結合使用時，其藉由將邊界延伸至所列數值之上與之下而調整該範圍。一般而言，術語「約」可將數值上下調整10%、向上或向下(更高或更低)。

【0085】 本文中所用的術語「主鏈」係指ISVD多肽或V_HH多肽胺基酸序列包括任一L_{1-2}連接子及延伸子，但排除掉延長部分且為了避免疑慮也排除掉了適於與該主鏈融合的任何延長部分。

【0086】 術語「結合親和力」係為兩個分子(例如ISVD，諸如V_HH片段)與抗原之間的非共價交互作用之強度的量度。該術語係用於描述單價交互作用。兩個分子(例如ISVD，諸如V_HH片段)間透過單價交互作用的結合親和力可藉由判定平衡解離常數(K_D)來定量。K_D可藉由測量複合物形成及解離之動力學來測定，例如藉由表面電漿子共振(SPR)方法。對應於單價複合物之締合及解離的速率常數

分別被稱為締合速率常數k_a（或k_{on}）及解離速率常數k_d（或k_{off}）。K_D透過方程式$K_D = k_d / k_a$而與k_a及k_d有關。

【0087】 按以上定義，與不同分子交互作用有關的結合親和力(諸如不同ISVD對於給定抗原的結合親和力)可藉由比較個別抗體/抗原複合物之K_D值而比較。

【0088】 解離常數之值可藉由廣為人知的方法直接測定。用於評估配體(諸如ISVD)對於靶標之結合能力的標準測定法係所屬技術領域中已知的，包括(例如) ELISA、西方墨點法、RIA、及流式細胞測量儀分析。ISVD之結合動力學及結合親和力亦可藉由所屬技術領域中已知的標準測定法（諸如SPR）評估。

【0089】 可以進行競爭性結合測定法，其中將ISVD (諸如V_HH)與靶標的結合跟存在該靶標之另一配體(諸如另一ISVD)的情況下對該靶標的結合進行比較。

【0090】 除非上下文相矛盾，較佳由本文所述之表面電漿子共振來判定K_D (見實例7)。

【0091】 較佳地，該能夠結合FIX(a)之ISVD的K_D值為3 μM或更低，諸如15 nM或更低，諸如11.7 nM或更低。

【0092】 較佳地，該能夠結合FX(a)之ISVD的K_D值為3 μM或更低，諸如350 nM或更低，諸如300 nM或更低。

【0093】 「跨物種反應性」ISVD (或V_HH片段)係以相當的親和力結合於來自所有所指示之物種（例如人類及食蟹獼猴）之(例如) FIX，特別是在係數為100之K_D範圍內，諸如在係數為50之K_D範圍內、在係數為20之K_D範圍內、或在係數為10之K_D範圍內。在既定係數X之K_D範圍內意謂對特別所列之物種的最高親和力係比對不同的所列物種之最低親和力高不超過X倍。本領域中熟習該項技藝者將瞭解，任何用於量測親和力之方法係可用於驗證跨物種反應性ISVD在本文

所述之既定K_D係數範圍內結合於來自於所有所列物種的靶標抗原，只要相同條件應用於所有所列物種之K_D量測即可。較佳地，使用SPR量測K_D值，尤其在25°C下。較佳地，使用跨物種反應性ISVD（諸如V_HH）來量測親和力。

【0094】 胺基酸為含有胺基及羧酸基且視情況含有一或多個通常稱為側鏈之額外基團的分子。

【0095】 術語「胺基酸」包含正準胺基酸（其為基因編碼的）及非天然胺基酸。非天然胺基酸之非限制性實例為Aib (α-胺基異丁酸)、去胺基組胺酸（替代名稱3-(咪唑-4-基)丙酸，縮寫為Imp (咪唑丙醯基)）、及正準胺基酸之d-異構物。未說明光學異構體之多肽內的所有胺基酸殘基在本文中應理解為是指l-異構體(除非另外指明)。

【0096】 術語「抗體」於本文中係指包含有或衍生自免疫球蛋白序列的蛋白質，其能夠結合至抗原或其部分。

【0097】 「抗體」包括(但不限於)包含至少四個多肽鏈的全長抗體：藉由雙硫鍵連接的二個重鏈(HC)及二個輕鏈(LC)，以及包含至少三個多肽鏈的抗體：藉由雙硫鍵連接的二個重鏈(HC)及一個輕鏈(LC)。一類的免疫球蛋白為IgG。於人類中，IgG類型基於其等之重鏈恆定區序列可被分成四個次類型IgG1、IgG2、IgG3及IgG4。輕鏈基於其等之序列組成之不同可被分成兩個類型：κ及λ鏈。IgG分子係由兩條重鏈(藉由二或多個雙硫鍵彼此連接)及兩條輕鏈(各藉由一個雙硫鍵附接至一重鏈)所構成。術語「抗體」亦涵蓋單域抗體，諸如V_HH片段及V-NAR片段。

【0098】 如本文所用之術語「高度變異區」係指抗體中負責抗原結合的胺基酸殘基。高度變異區包含「互補性決定區」，也稱為「CDR」。

【0099】 「框架」或「FR」區域為高度變異區以外的可變結構域區域。因此，一個抗體或ISVD從N端到C端包括FR1、CDR1、FR2、CDR2、FR3、CDR3

及FR4結構域。重鏈的CDR3為通常對抗原結合貢獻最大的區域。

【0100】 如本文所用之術語「表位」係指能夠特異性結合至抗體之蛋白質決定位(determinant)。表位通常由分子(諸如胺基酸或糖側鏈)之表面元件所組成，且通常具有特定三維結構特性以及特定電荷特性。構形及非構形表位的差別在於與前者的結合在能夠破壞蛋白質的結構之變性劑的存在下即失去，但後者並不會。

【0101】 於藉由ISVD（V_HH片段）及其靶標間的複合物之空間坐標定義的衍生自X射線的結晶結構之前後文中，除非另加具體指出或與前後文矛盾，術語表位於本文中的具體定義為其特徵為在離FIX/FIXa或FX/FXa中的重原子（即非氫原子）4 Å的距離內具有重原子的ISVD殘基。

【0102】 給定ISVD/抗原對之表位可藉由例行方法鑑認，諸如以下實例中所述者。例如，可將ISVD及抗原結合並可結晶出該ISVD/抗原複合物。可測定複合物之結晶結構及將其用於鑑認該ISVD及其抗原間的交互作用之特定位置。

【0103】 在一實施例中，本文所揭露之ISVD多肽衍生物係包含第一ISVD及第二ISVD，該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260中之至少一者的因子FIX (SEQ ID NO:1)或其活化型上的表位，而該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）中之至少一者的FX (SEQ ID NO:2)上的表位。

【0104】 在一實施例中，該ISVD多肽衍生物係包含第一ISVD及第二ISVD，該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）中之至少一者的因子IX (SEQ ID NO:1)或其活化型上的表位，而

該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178

（連續編號）中之至少一者或是包含有胺基酸殘基N173、P174、F175、L177、L178及D179中之至少一者的因子X (SEQ ID NO:2)上的表位。

【0105】 凝血因子IX (FIX)為維生素K依賴性凝血因子，其結構類似於因子VII、凝血酶原、因子X、及蛋白質C。FIX於血漿中以單鏈酶原(SEQ ID NO:1)的形式循環。其循環性酶原形式係由分成包含以下者的四個明顯不同的域的415個胺基酸所組成：N端富γ-羧基麩胺酸(Gla)域、兩個EGF域及C端類胰蛋白酶絲胺酸蛋白酶域。FIX之活化係藉由釋放活化胜肽(SEQ ID NO:1之殘基146至180)的於Arg145及Arg180的限制性蛋白水解發生。因此，經活化FIX(FIXa)係由SEQ ID NO:1之殘基1-145(輕鏈)及SEQ ID NO:1之殘基181-415(重鏈)構成。

【0106】 循環性FIX分子因此包含FIX酶原及FIX之活化型，其等關於SEQ ID NO:1於本文中總體被稱為FIX及FIXa。

【0107】 經活化因子IX被稱為因子IXa或FIXa。術語「FIX(SEQ ID NO:1)及/或其活化型FIXa」亦可被稱為「FIX/FIXa」或僅稱為「FIX(a)」。

【0108】 FIXa為類胰蛋白酶絲胺酸蛋白酶，其藉由於凝血期間產生支持適當的凝血酶形成所需的因子Xa之大部分(作為tenase複合物之部分)而於止血扮演關鍵性角色。

【0109】 FIX於本文中係以對應於人類FIX之Ala148對偶基因形式的SEQ ID NO:1代表(Anson等人，EMBO J. 1984 3:1053-1060; McGraw等人，Proc Natl Acad Sci USA. 1985 82:2847-2851; Graham等人，Am. J. Hum. Genet. 1988 42:573-580)。於本發明中，FIX意欲涵蓋所有FIX之天然變體，諸如T148變體(Uniprot ID P00740)。

【0110】 FX係維生素K依賴性凝血因子，其結構類似於因子VII、凝血酶原、FIX、及蛋白質C。FX於血漿中以包括SEQ ID NO:2之殘基1-139(輕鏈)及SEQ ID NO:2之殘基143-448(重鏈)的二鏈酶原的形式循環。人類FX酶原包括了分別包

括有N端富伽瑪-羧基麩胺酸(Gla)域（殘基1-45）、兩個EGF域、EGF1（殘基46-82）及EGF2（殘基85-125）的四個不同的域，以及C端類胰蛋白酶絲胺酸蛋白酶域（殘基195-448）。FX之活化係藉由造成活化胜肽(殘基143-194)之釋放的於Arg194的限制性蛋白水解發生。因此，經活化FX (FXa)係由SEQ ID NO:2之殘基1-139(輕鏈)及SEQ ID NO:2之殘基195-448(經活化重鏈)構成。循環的FX分子因此包含FX酶原及FX之活化型，其等關於SEQ ID NO:2於本文中分別被稱為FX及FXa。於本發明中，FX意欲涵蓋所有FX之天然變體。術語「FX (SEQ ID NO:2)及/或其活化型(FXa)」亦可被稱為「FX/FXa」或「FX(a)」。

【0111】 如本文所用之術語「保守性取代」係指胺基酸可以被取代成具有相似生物化學性質的胺基酸的情況，例如，鹼性胺基酸可以被取代成另一個鹼性胺基酸(例如離胺酸取代成為精胺酸)，酸性胺基酸可以被取代成另一個酸性胺基酸(例如麩胺酸取代成天門冬胺酸鹽)，中性胺基酸可以被取代成另一個中性胺基酸(例如蘇胺酸取代成絲胺酸)，帶電荷的胺基酸可以被取代成另一個帶電荷的胺基酸(例如麩胺酸取代成離胺酸)，親水性胺基酸可以被取代成另一個親水性胺基酸(例如天門冬醯胺取代成麩醯胺)，疏水性胺基酸可以被取代成另一個疏水性胺基酸(例如丙胺酸取代成纈胺酸)，極性胺基酸可以被取代成另一個極性胺基酸(例如絲胺酸取代成蘇胺酸)，芳香族胺基酸可以被取代成另一個芳香族胺基酸(例如苯丙胺酸取代成色胺酸)，且脂族胺基酸可以被取代成另一個脂族胺基酸(例如白胺酸取代成異白胺酸)。

【0112】 如本文所用之術語「賦形劑」係指添加到醫藥組成物中的惰性物質，用以進一步促進活性成分的投予。

【0113】 賦形劑的實例包括(但不限於)碳酸鈣、磷酸鈣、各種糖類及各類澱粉、L-精胺酸、菸鹼醯胺、SNAC、纖維素衍生物、明膠、蔬菜油及聚乙二醇。

【0114】 如本文所用之術語「延伸子」(E)係指適於接附至ISVD多肽的肽

或多肽。

【0115】 該延伸子可以是幾個胺基酸、1至10個胺基酸，可以更長，10至30個胺基酸，或者可以很長，超過30個胺基酸。延伸子最好存在於ISVD多肽、ISVD多肽衍生物、V_HH多肽或V_HH多肽衍生物的N端（N端延伸子）或C端（C端延伸子），或同時存在。或者，延伸子接附在CDR序列之外的ISVD多肽的其他地方，諸如在一或多個框架區域或連接子L_{1-2}中。

【0116】 延伸子較佳地係重組融合至ISVD多肽。在其他實施例中，延伸子係與ISVD多肽共軛。

【0117】 非限制性實例包括：具有由GQACPC (SEQ ID NO:9)構成之6個胺基酸C端延伸子的化合物#22、具有由GGGGCSCHHHHHH (SEQ ID NO:8)構成之13個胺基酸C端延伸子的化合物#6、以及由GGGGSHHHHHH (SEQ ID NO: 7)構成之11個胺基酸C端延伸子的化合物#71。

【0118】 該延伸子之目的係提供延長部分的接附點及/或提供一種純化手段。因此，術語延伸子並不涵蓋延長子及延長部分。

【0119】 例如，包括有任何重組融合至該多肽N端或C端之延長連接子(L_P)連接子的延長子並不視為是延長子。

【0120】 如本文所用之術語「融合」係指符合讀框地連接最初編碼獨立的蛋白質或多肽或其片段之二或更多個DNA序列。融合多肽DNA序列的轉譯將產生一個單一的多肽序列，該序列可能具有源自每個原始蛋白質或肽的功能特性。編碼融合蛋白的DNA序列可以通過標準的分子生物學方法，諸如重疊PCR或DNA連接來人工創建。所得的融合多肽DNA序列可插入適當的表現載體中，該載體支持異源融合蛋白在宿主生物體（諸如細菌、酵母菌、真菌、昆蟲細胞或哺乳動物細胞）中的表現。延長部分可(例如)與ISVD多肽或V_HH多肽主鏈的C端或N端融合。

【0121】 如本文所用之術語「宿主細胞」包括任何種類的細胞系統，其可經工程改造以生成本文所揭露的ISVD。宿主細胞包括(但不限於)培養的細胞，例如哺乳動物培養細胞，諸如CHO細胞、HEK293T細胞、BHK細胞、NSO細胞、SP2/0細胞、YO骨髓瘤細胞、P3X63小鼠骨髓瘤細胞、PER細胞、PER.C6細胞或雜交瘤細胞、酵母菌細胞、真菌細胞以及昆蟲細胞。

【0122】 本領域中已知的術語「一致性」係指如通過比較序列所判定之二或多個多肽序列之間的關係。在本領域中，「一致性」亦意指如由二或多個胺基酸殘基串之間的匹配數所判定之多肽之間的序列相關性程度。「一致性」係測量具有通過特定數學模型或電腦程式（即「演算法」）解決的缺口比對（如果有的話）之二或多個序列中之較小者的相同匹配百分比。相關的多肽之一致性可藉由已知的方法輕易計算。本發明中之相似度及一致性係使用來自於軟體EMBOSS-6.6.0之程式Needleman (Needleman等人, J. Mol. Biol. 1970; 48:443-453)判定，且分別使用10及0.5做為缺口開放(gaps opening)及缺口延伸(gaps extensions)的參數（缺口開放(gapopen)=10，缺口延伸(gapextend)=0.5）。

【0123】 術語「免疫球單可變結構域」或「ISVD」係作通用術語使用，分別包括抗原結合結構域或片段，諸如VH及VL結構域。

【0124】 因此，ISVD可以是輕鏈可變結構域序列（如VL序列），或重鏈可變結構域序列（如VH序列）；例如，源自習知四鏈抗體中之重鏈可變結構域序列或源自習知四鏈抗體中之輕鏈可變結構域序列。

【0125】 一個特殊類型的ISVD為V_HH片段，其最初被鑑定為一種免疫球蛋白，其定義為由單個單體可變抗體結構域組成的抗體片段。V_HH片段是一種單域抗體，其涵蓋了僅重鏈之抗體的抗原結合可變區，其可獲自駱駝科動物。V_HH片段的大小約為15 kDa。它們含有一個單鏈分子，可以用一個單一結構域來結合其同源抗原。V_HH片段的抗原結合表面通常比習知抗體的表面更凸出（或突出），

後者通常是平坦或凹陷的。V_HH片段係由四個框架區域(或FR)及三個互補性決定區(或CDR)組成，前者的序列及結構被定義為保守的，後者在序列內容及結構構象上都顯示出高度的可變性，且其涉及抗原結合並提供抗原特異性。

【0126】 另一種類型的ISVD可獲自軟骨魚的IgNAR及其被稱為「V-NAR片段」的單域抗體。

V_HH及V-NAR片段不包含恆定結構域，因此沒有Fc區域，而Fc區域通常是部分/全長及/或經工程改造/天然僅重鏈抗體的一部分[Dooley等人 (2006) Dev. Comp. Immunol. 30:43–56; Muyldermans S. (2013) Annu Rev Biochem. 82:775-97]。

【0127】 除此之外，關於駱駝科動物V_HH片段以及其生產及/或分離及/或使用方法的一般描述可在以下參考文獻WO94/04678及WO97/49805中找到。關於來自軟骨魚的重鏈免疫球蛋白及其可變域以及其生產及/或分離及/或使用的方法的一般描述係可在WO2005/118629中找到。

【0128】 V_HH片段中的胺基酸殘基總數通常在110至140範圍內。然而，應注意的是，若V_HH的部分、片段或類似物之長度及/或大小符合下文概述的進一步要求且亦優選適用於本文所述之目的，則這類部分、片段或類似物不受特別限制。V_HH片段的分子量通常在12-15kDa之間。V_HH片段的pI通常是鹼性的，就像抗體一般，意味著pI值一般在7以上，通常在7.5和8.5之間。V_HH片段通常涵蓋了一般由位於框架區域1及3中之保守半胱胺酸對所形成的至少一雙硫鍵。這類雙硫鍵確保了V_HH片段正確摺疊與穩定性，且若(例如)側鏈修飾及/或共軛係以其靶向V_HH片段中所導入之未成對半胱胺酸的方式進行，則期望能保留這類的雙硫鍵。

【0129】 在本文件中，ISVD的CDR序列（諸如V_HH片段）係使用Kabat定義來判定的(Kontermann及Dübel, 2010編, Antibody Engineering, 第2卷, Springer Verlag Heidelberg Berlin, Martin, 第3章, 第33-51頁)。根據這方法，可變結構域的

CDR的定義為位置31-35 (CDR1)、位置50-65 (CDR2)及位置95-102 (CDR3)。

【0130】 然而，當提到本文所述之多肽化合物中之特定胺基酸殘基位置包括ISVD多肽或V_HH多肽中的CDR及框架(FR)區域時，，除非另有說明，否則使用連續的編號。

【0131】 如本文所用之術語「ISVD多肽」係指由二或更多個ISVD（諸如一第一ISVD (ISVD1)及一第二ISVD (ISVD2)）組成的多肽，其例如由任何適當成分及長度的連接子(L_{1-2})連接，或完全不需要任何連接子而作為直接結構域融合。

【0132】 如本文所用之術語「連接子」係指至少一個在化學實體之間形成共價鍵的原子。若該化學實體僅通過肽鍵連接，則可將連接子稱為「肽連接劑」。否則，該連接子可被稱為「化學連接子」。

【0133】 ISVD多肽的一實例為兩個經由一連接子(L_{1-2})所連接的ISVD。

【0134】 ISVD多肽的另一實例為兩個經由進一步包含有一或多個延伸子的連接子(L_{1-2})所連接的ISVD。

【0135】 ISVD多肽的另一實例為兩個沒有連接子(L_{1-2})連接的ISVD，且其進一步包含一或多個延伸子，諸如(但不限於) C端及/或N端延伸子。

【0136】 連接子(L_{1-2})可例如由一個包括無重複或多重複之胺基酸序列所組成。

【0137】 例如，連接子可包含2至50個胺基酸、5至40個胺基酸、或10至30個胺基酸。

【0138】 連接子的非限制性實例包括*-GGGGS-*連接子、*-GQAPGQ-*連接子(SEQ ID NO:20)、*-QAPGQA-*連接子(SEQ ID NO:16)、*-GI-*連接子、*-GV-*連接子、*-GT-*連接子、*-GL-*連接子、或另一種組成連接子。兩個連接子實例為x2及x6重複組成的GGGGS，其長度分別為10及30個胺基酸殘基。

【0139】 為了避免疑慮，L_{1-2}中的下標1-2並非意味著所連接之ISVD的特定方向，即L_{1-2}可(例如)連接ISVD1至ISVD2，或連接ISVD2至ISVD1 (N端到C端)。

【0140】 SEQ ID NO:14-24係表示連接子L_{1-2}的非限制性實例。

【0141】 在一些實施例中，ISVD多肽包含下式中任一者概述的延伸子(E)。

ISVD1 – ISVD2，或

ISVD1 – ISVD2 – E，或

ISVD2 – ISVD1 – E，或

E - ISVD1 – ISVD2，或

ISVD1 – L_{1-2} – ISVD2 – E，或

ISVD2 – L_{1-2} – ISVD1 – E，或

E - ISVD1 – L_{1-2} – ISVD2。

【0142】 該延伸子(E)例如可連接到下式中任一者概述的ISVD多肽上（N至C端）：

ISVD1 – ISVD2，或

ISVD1 – ISVD2 – E，或

ISVD2 – ISVD1 – E，或

E - ISVD1 – ISVD2，或

ISVD1 – L_{1-2} – ISVD2 – E，或

ISVD2 – L_{1-2} – ISVD1 – E，或

E - ISVD1 – L_{1-2} – ISVD2。

【0143】 ISVD多肽較佳地包含一個延長部分。在這情況下，ISVD多肽被稱為是ISVD多肽衍生物。

【0144】 在一些實施例中，該第一ISVD係作為一或多個延長部分的接附

點。

【0145】 在一些實施例中，該第二ISVD係作為一或多個延長部分的接附點。

【0146】 在一些實施例中，該L_{1-2}連接子係作為一或多個延長部分的接附點。

【0147】 為了避免疑慮，當延長部分包括一個連接子(Lp)接附到延伸子(E)而形成ISVD1-L_{1-2}-ISVD2時，連接子L_P並不被認為是該「延伸子」的一部分。

【0148】 在一些態樣中，ISVD多肽與非ISVD（例如小的化學非多肽分子、碳水化合物、脂肪酸或寡肽或多肽或蛋白質，諸如抗體或較佳為抗體片段）進行化學共軛。

【0149】 在一實施例中，ISVD多肽係未使用連接子而連接至IgG抗體的Fc結構域。

【0150】 在另一實施例中，ISVD多肽係藉由一連接子而連接至IgG抗體之Fc結構域或其片段。

【0151】 在較佳實施例中，延伸子係融合至ISVD多肽且因而未以化學共軛方式連接至ISVD多肽。

【0152】 在一些實施例中，延長部分係融合至ISVD多肽且因而未以化學共軛方式連接至ISVD多肽。

【0153】 如本文所用之術語「等電點」或「pI」係指蛋白質（諸如抗體）的總淨電荷為零時的pH值。在蛋白質中可能有許多帶電基團，且在等電點時所有這些電荷的總和為零。在pH值高於等電點時，蛋白質的總淨電荷為負，而在pH值低於等電點時，蛋白質的總淨電荷為正。

【0154】 pI可以是理論上的，也可以是實驗上確定的等電點。

【0155】 熟習該項技藝者知道確定蛋白質等電點的方法。最常見的是，蛋

白質的等電點是根據蛋白質的胺基酸序列計算的。有許多（線上）工具可以確定蛋白質的等電點，諸如「ExPASy Compute pI/Mw」，參見Protein Identification and Analysis Tools on the ExPASy Server; Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A.; (In) John M. Walker (編): The Proteomics Protocols Handbook, Humana出版社(2005)，第571-607頁。較佳地，使用Skoog & Wichman, 1986的演算法及胺基酸殘基的pKa來計算pI。

【0156】 pl也可以通過實驗來確定，例如使用基於電荷的分離技術（諸如等電聚焦(IEF)凝膠電泳、毛細管等電聚焦(cIEF)凝膠電泳）來分離電荷變體。

【0157】 在一實施例中，ISVD多肽中之第一及第二ISVD為V-NAR片段且這類化合物被稱為「V-NAR多肽」。

【0158】 在另一實施例中，ISVD多肽中的第一及第二ISVD為V_HH片段且這類化合物被稱為「V_HH多肽」。在一這類實施例中，第一V_HH片段能夠結合FIX/FIXa，而第二V_HH片段能夠結合FX/FXa。在一較佳實施例中，V_HH多肽為雙特異性V_HH多肽。

【0159】 為了避免疑慮，術語多特異性、三特異性或雙特異性旨在反映被ISVD（諸如V_HH片段）結合的抗原數量，即不包括被延長部分（如果存在）結合的分子，諸如(但不限於)白蛋白。

【0160】 在一實施例中，不具延長部分之V_HH多肽的分子量在27至29 kDa的範圍內。

【0161】 在一較佳實施例中，包括有一或多個延長部分及可選的一或多各延伸子之VHH多肽（V_HH多肽衍生物）的分子量在28至33 kDa的範圍內。

【0162】 本領域中熟習該項技藝者將理解，上述實施例同樣也適用於V-NAR多肽。

【0163】 如本文所用之術語「游離半胱胺酸」係指多肽鏈中可用於反應(例

如化學共軛）的半胱胺酸殘基，因而不是天然或工程改造的內部雙硫鍵的一部分。實質上，游離半胱胺酸可用於共軛，儘管游離半胱胺酸殘基（包括重組導入的游離半胱胺酸）於宿主細胞中重組表現多肽的期間，經常被小硫醇（諸如半胱胺酸、同型半胱胺酸或麩胱甘肽）阻斷。在ISVD多肽的重組產生中也可觀察到已導入一或多個游離半胱胺酸這一點。因此，使用適當的還原劑（諸如雙(對磺酸根苯基)苯基膦二水合物或三(2-羧基乙基)膦鹽酸鹽）進行還原反應，可用來釋放及製備游離半胱胺酸供與所要部分（諸如(但不限於)延長部分，諸如C18二酸γ-Glu 2xOEG脂肪酸部分）共軛。

【0164】 本文所揭露之ISVD多肽（諸如V_HH多肽）可為多特異性的，諸如(但不限於)雙特異性或三特異性。

【0165】 如本文所用之術語「雙特異性ISVD多肽」或「雙特異性V_HH多肽」係分別指能夠與兩種不同的抗原或相同抗原上的兩種不同表位結合的ISVD多肽或V_HH多肽。

【0166】 如本文所用之術語「三特異性ISVD多肽」或「三特異性V_HH多肽」係分別指能夠與三種不同的抗原或相同抗原上的三種不同表位或存在於兩種不同抗原上的三種不同表位結合的ISVD多肽或V_HH多肽。

【0167】 如本文所用之術語「多特異性ISVD多肽」或「多特異性V_HH多肽」係分別指能夠與二或更多個不同抗原或相同抗原上的二或更多個不同表位結合的ISVD多肽或V_HH多肽。因此，多特異性ISVD多肽或多特異性V_HH多肽分別包含雙及三特異性ISVD多肽或V_HH多肽。

【0168】 本領域中熟習該項技藝者將理解，上述內容也適用於多肽衍生物（即包括延長部分）。

【0169】 如本文所用之術語「口服生物可利用率」或「經口生物可利用率」係指所投予藥物在口服投予後的量（以所投予藥物之血漿濃度與時間曲線下的

面積進行估計）相對於靜脈投予所述藥物之後於全身循環中的所投予藥物數量。

【0170】 本文所用之術語「互補位」係指該ISVD上之抗原特異性地與之結合的地區或區域，即其以之與該抗原作物理接觸。

【0171】 於藉由ISVD（V_HH片段）及其靶標間的複合物之空間坐標定義的衍生自X射線的結晶結構之前後文中，除非另加具體指出或與前後文矛盾，術語互補位於本文中的具體定義為其特徵為在離FIX/FIXa或FX/FXa中的重原子（即非氫原子）4 Å的距離內具有重原子的ISVD殘基。

【0172】 給定ISVD/抗原對之互補位(及表位)可藉由例行方法鑑認，諸如以下實例中所述者。例如，可將ISVD及抗原結合並可結晶出該ISVD/抗原複合物。可測定複合物之結晶結構及將其用於鑑認該ISVD及其抗原間的交互作用之特定位置。

【0173】 術語「醫藥學上可接受的賦形劑」係指有益於製備醫藥組成物的賦形劑，其通常是安全的、無毒的，且為人類醫藥用途可接受的。這類賦形劑可例如為固體、液體或半固體。

【0174】 本文所使用之術語「血漿半衰期」意指藉由正常生物過程從患者血清或血漿中代謝或消除投予患者之物質的一半量所需之時間。

【0175】 如本文所用之術語「優先結合」係應理解為ISVD（諸如V_HH片段）上的結合區與一組分（例如活化的FIX）的結合係優先於或有利於另一組分。因此，「優先結合」不一定需要排他性地結合或不可檢測地結合另一組分。例如，相較於未活化的FIX，抗FIX(a) ISVD（諸如抗FIX(a) V_HH片段）係可優先與活化的FIX結合。

【0176】 術語「促凝血抗體」係指例如藉由加速血液凝固過程及/或增加一或多種凝血因子的酶活性而增強血液凝固的抗體。

【0177】 本文所使用之術語「促凝血活性」係指化合物（諸如抗體）例如

藉由加速血液凝固過程及/或增加一或多種凝血因子的酶活性而增強血液凝固的能力。因此，術語「促凝血劑活性」係涵蓋(但不限於)以下所列的一或多種活性：

增強因子IXa介導之因子X活化係基於FIXa介導的FX活化藉由醯胺（顯色或發螢光）測定。該測定法係通過裂解FXa特異性肽受質而測量FXa。受質生成，其產生可利用吸光度進行光度測定的顏色。

縮短凝血時間，如利用凝血測定法（諸如活化部分凝血激酶時間(APTT)）測量固有活性及常見凝血途徑。將血漿與含有接觸活化劑（例如土耳其鞣酸(ellagic acid)或高嶺土）及磷脂質之APTT試劑預處理。添加氯化鈣以促進纖維蛋白凝塊形成。可能的讀數為凝血時間或凝塊波形。

增進凝血酶生成，如凝血酶生成試驗（諸如校正式自動化血栓造影(CAT)）之測定。血栓圖描述凝血酶在凝血血漿中之濃度，因此為止血系統的功能測試。本試驗係基於螢光測定，其係隨時間推移利用凝血酶切割螢光受質Z G G R AMC而產生。亦見本文實例8中所使用的方法。

增進血塊形成的整體黏彈特性，如在全血中之剪切應力下，利用黏彈性止血法（諸如ROTEM (旋轉血栓彈性描記法)測定。在儀器中，滾珠軸承銷在固定杯中旋轉。在凝集過程中，樣本中的纖維蛋白絲會在杯壁與銷之間形成，且絲的強度會影響銷的運動，並檢測此現象。

縮短全血關閉時間(WBCT)，如血小板功能分析儀，其係基於血小板活化後馮威里氏因子(VWF)介導之血小板對膠原蛋白的黏附。產生高剪切應力，導致血小板黏附與血小板凝集。測定血流從開始到結束的時間。

【0178】 本文所揭露之ISVD多肽較佳地包含一、二、三或四個「延長部分」(PM)。更佳地為一或二個延長部分。

【0179】 如本文所用之術語「延長部分」係指具有延長半衰期特性的部分，包含有一「延長子」(P)及一可選的「連接子」(L_P)。因此，術語「延長」係指的

是半衰期的延長，而延長子或延長部分因此用作延長本文所揭露之ISVD多肽的半衰期的目的。

【0180】 延長部分(PM)係包含一延長子「P」及一可選的連接子(L_P)。

【0181】 每個延長部分較佳地係附接該化合物多肽主鏈中的一個表面暴露離胺酸或半胱胺酸殘基。附接點通常稱作為R1（在連接超過一個延長部分的情況下則稱為R2、R3等，其中R1 ≠ R2 ≠ R3等）。

【0182】 熟習該項技藝者將能夠鑑認出適合接附的其他表面暴露殘基。

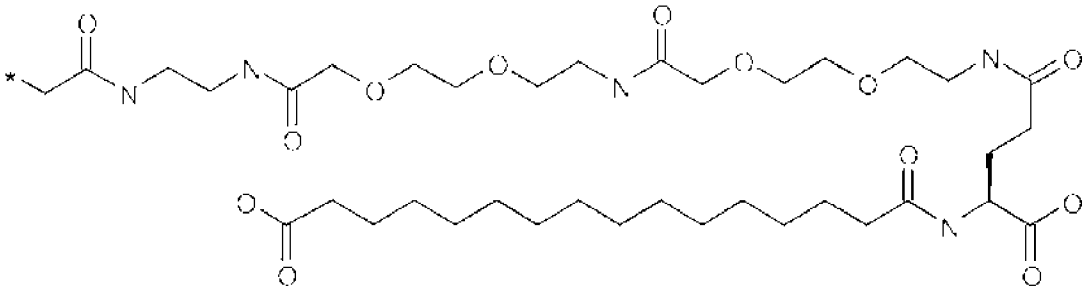
【0183】 延長部分可由一個延長子所組成。

【0184】 延長部分可包含一個連接子(L_P)及一個延長子(P)。

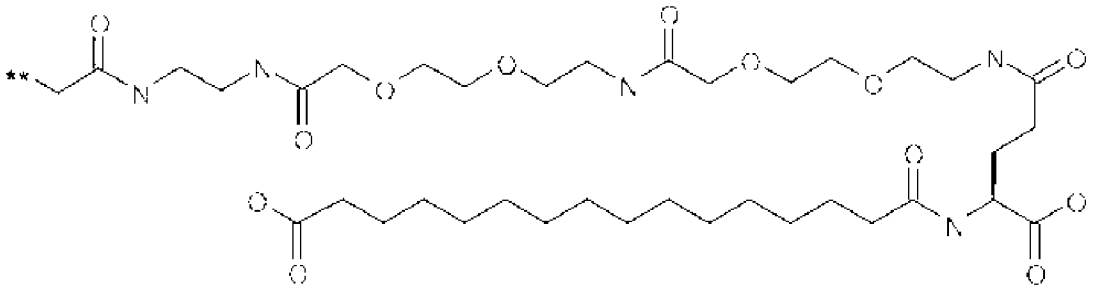
【0185】 延長部分可包含一個連接子及二或更多個延長子。

【0186】 當連接子(L_P)存在時，該延長部分經由L_P接附至ISVD多肽主鏈。當連接子(L_P)不存在時，P接附至該多肽主鏈。

【0187】 在一實施例中係存有一第一延長部分及一第二延長部分，其中所述第一延長部分包含以下結構：



且其中所述第二延長部分包含以下結構：



其中「*」(R1)係表示該ISVD多肽之第一延長部分的接附點，且

其中「**」(R2)係表示該ISVD多肽之第二延長部分的接附點，

其中「*」為SEQ ID NO:629之位置257的Cys，且

其中「**」為SEQ ID NO:629之位置259的Cys。

【0188】 本文所揭露之ISVD多肽可包含單個半胱胺酸或離胺酸殘基，其以單個延長部分與其接附/共軛。

【0189】 本文所揭露之ISVD多肽可包含兩個離胺酸殘基及兩個延長部分。

【0190】 本文所揭露之ISVD多肽可包含兩個離胺酸殘基及兩個相同的延長部分。

【0191】 本文所揭露之ISVD多肽可包含兩個離胺酸殘基及兩個不相同的延長部分。

【0192】 本文所揭露之ISVD多肽可包含三個離胺酸殘基及三個延長部分。

【0193】 本文所揭露之ISVD多肽可包含三個離胺酸殘基及三個相同的延長部分。

【0194】 本文所揭露之ISVD多肽可包含三個離胺酸殘基及三個不相同的延長部分（例如，第一及第二延長部分可以是相同的，而第三延長部分則與所述第一及第二延長部分不同）。

【0195】 本文所揭露之ISVD多肽可包含兩個半胱胺酸殘基及兩個延長部分。

【0196】 本文所揭露之ISVD多肽可包含兩個半胱胺酸殘基及兩個相同的延長部分。

【0197】 本文所揭露之ISVD多肽可包含兩個半胱胺酸殘基及兩個不相同的延長部分。

【0198】 本文所揭露之ISVD多肽可包含三個半胱胺酸殘基及三個延長部分。

【0199】 本文所揭露之ISVD多肽可包含三個半胱胺酸殘基及三個相同的延長部分。

【0200】 本文所揭露之ISVD多肽可包含三個半胱胺酸殘基及三個不相同的延長部分（例如，第一及第二延長部分可以是相同的，而第三延長部分則與所述第一及第二延長部分不同）。

【0201】 其中該ISVD多肽包含二或三個延長部分，該等延長部分較佳為近似的，更佳為實質上相同的，或最佳為相同的。

【0202】 在諸如本文所揭露之延長部分的化學部分的上下文中，可使用本領域中已知任何適宜電腦程式及/或演算法來判定相似度及/或一致性。

【0203】 包括有延長部分之化合物可被稱為為「衍生物」。例如，「ISVD多肽衍生物」可以理解為一種包含有延長部分的ISVD多肽，一種包含有延長部分的「V_HH多肽衍生物」V_HH多肽，及一種包含有延長部分的「V-NAR多肽衍生物」。

【0204】 延長部分能夠以非共價方式結合白蛋白，藉以促進ISVD多肽衍生物在血流中循環並延長其半衰期。因此，在一實施例中該延長部分為白蛋白結合部分。

【0205】 該延長子(P)可包含一醯基。該醯基可為分枝的或未分枝。該醯基

可為飽和的或不飽和。該延長子可包含一脂肪醯基。該醯基可為分枝的或未分枝。該醯基可為飽和的或不飽和。

【0206】 該延長子可包含一遠端羧酸基團。

【0207】 該延長子可包含一脂肪酸基團。

【0208】 該延長子可包含一脂肪酸基團及一醯胺基。

【0209】 該延長子可包含一遠端羧酸基團及一醯胺基。

【0210】 該延長子可包含一烷基。

【0211】 該延長子可包含一芳基。

【0212】 該延長子可包含一四唑基。

【0213】 該延長子可包含一磺酸基。

【0214】 該延長子可包含一苯氧基。

【0215】 該延長子可包含一苯甲酸基團。

【0216】 該延長子可包含8-30個碳原子。該延長子可包含8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29或30個碳原子。

【0217】 該延長子可包含6-30個連續-CH_2-基團。該延長子可包含一包含有至少6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29或30個連續-CH_2-基團之碳鏈。

【0218】 該延長子可包含12-26個碳原子。該「延長子」可包括12、13、14、15、16、17、18、19、20、21、22、23、24、25或26個碳原子。

【0219】 該延長子可包括10-26個連續-CH_2-基團。該延長子可包含一包含有至少10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25或26個連續–CH_2-基團之碳鏈。

【0220】 該延長子可包含16-22個碳原子。

【0221】 該延長子可包含14-20個連續-CH_2-基團。該延長子可包含一包含有14、15、16、17、18、19或20個連續-CH_2-基團之碳鏈。

【0222】 該延長子可包含16-22個連續碳原子及14-20個連續-CH_2-基團。

【0223】 該延長子可包含16個連續碳原子及14個連續-CH_2-基團。

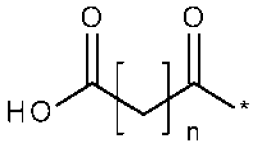
【0224】 該延長子可包含18個連續碳原子及16個連續-CH_2-基團。

【0225】 該延長子可包含20個連續碳原子及18個連續-CH_2-基團。

【0226】 該延長子可包含22個連續碳原子及20個連續-CH_2-基團。

【0227】 在一些實施例中該延長子為以下所定義的基團：

化學式a: HOOC-$(CH_2)_n$-CO-*，其中n為8-30範圍內的整數，其還可被稱為C(n+2)二酸或者被稱為

化學式a1: ，其中n為8-30範圍內的整數。

【0228】 該延長子可包含寡肽。在一實施例中，該延長子寡肽為10至40個胺基酸，諸如10至30個胺基酸，諸如15至25個胺基酸，且較佳地為20個胺基酸。該延長子寡肽序列組成可例如為SEQ ID NO:3及4中例示的融合序列QRLMEDICLPRWGCLWEDDF (SEQ ID NO:736) [亦見WO01/45746 A2]。例如，化合物#71包含了一個由QRLMEDICLPRWGCLWEDDFGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 4)所組成的50個胺基酸N端延長部分，其中殘基1-20為延長子(P)，而殘基21-50為連接子(L_P)。

【0229】 該延長子可經由一胺基酸連接子L_P而接附至ISVD或ISVD多肽。

【0230】 在一實施例中，L_P可將延長子(P)連接至ISVD多肽主鏈之離胺酸或半胱胺酸殘基的側鏈。

【0231】 該ISVD多肽衍生物可包含兩個延長部分，其每一者包含14、15、

16、17、18、19或20個碳原子。該ISVD多肽可包含兩個延長部分，其中各個延長子(P)包含12、13、14、15、16、17或18個連續-CH_2-基團。

【0232】 該ISVD多肽衍生物可包含兩個C14二酸、兩個C16二酸或兩個C18二酸。

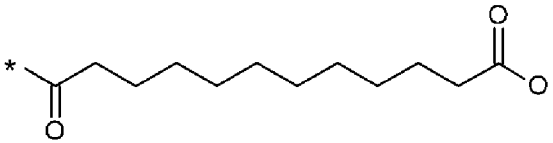
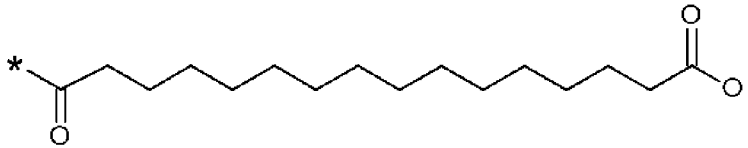
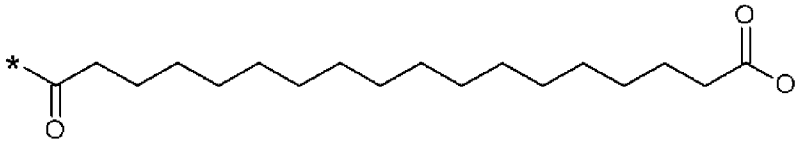
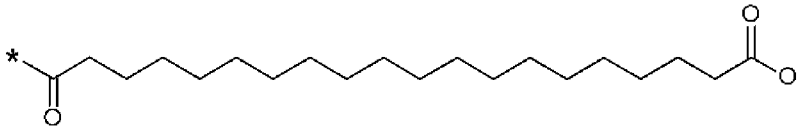
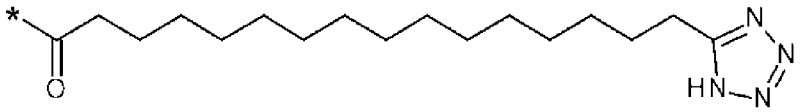
【0233】 該ISVD多肽衍生物可包含三個延長部分，其各包含一包含有12、13、14、15、16、17、18、19或20個碳原子的延長子。該ISVD多肽可包含三個延長部分，其中該延長子包含10、11、12、13、14、15、16、17或18個連續-CH_2-基團。

【0234】 為了避免疑慮，延長部分即使能夠結合(例如)白蛋白，但就本文所揭露之ISVD多肽是否為雙、三或多特異性的定義而言，其並不被認為是一種結合子。

【0235】 在一些實施例中，本文所揭露之ISVD多肽可包含一選自表1中所繪示之其中一者的延長子。

【0236】 在表2中，L_P係表示將繪示的延長子(P)連接至ISVD多肽主鏈的可選連接子。R1係表示ISVD多肽中的接附位點。

表1：表1：延長子(P)之實例（「*」係表示與L_P或R1的接附點）

延長子 ID	*結構*	*所使用化合物*
P1	 C12 二酸	化合物#65
P2	 C16 二酸	化合物#6、#7、#8、#9、#10、#11、#12、#13、#14、#15、#16、#17、#18、#19、#20、#21、#22
P3	 C18 二酸	化合物#4、#5、#24、#26、#28、#30、#32、#34、#36、#38、#40、#58、#59、#60、#61、#62、#63、#66
P4	 C20 二酸	化合物#67
P5		化合物#68

	四唑	
P6	QRLMEDICLPRWGCLWEDDF 白蛋白結合寡肽，SEQ ID NO:3 及 4 中例示為融合序列。 SEQ ID NO:3 係描述該白蛋白結合寡肽與一個 10 個胺基酸長的甘胺酸-絲胺酸連接子，而 SEQ ID NO:4 係描述該白蛋白結合寡肽與一個 30 個胺基酸長的甘胺酸-絲胺酸連接子。	化合物#70、#71、#72、#73

【0237】 在一實施例中該延長子係直接共軛在該ISVD多肽上，即不使用連接子L_P（即藉由共價鍵方式）。

【0238】 在其他實施例中該延長子係使用連接子L_P直接共軛在該ISVD多肽上。提供非限制性實例如下。

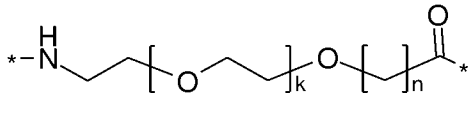
【0239】 該連接子L_P於存在時可包含Ado、Aeep或Aeeep、磺醯胺、Trx、ε-Lys、Ahx、Glu、γGlu、Gly、Ser、Ala及/或Thr。

【0240】 該連接子L_P可包括至少一個由以下化學式表示的部分：

化學式1：*-NH-$(CH_2)_2$-(O-(CH_2))$_k$-O-$(CH_2)_n$-CO-*

或

化學式2：



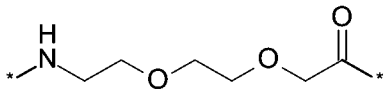
，

其中k為1-5範圍內的整數，且n為1-5範圍內的整數。

當k=1且n=1，該連接子元件可稱為Ado，或8-胺基-3,6-二氧雜辛醯基，其可由以下化學式表示：

化學式3:*-NH-$(CH_2)_2$-O-$(CH_2)_2$-O-CH_2-CO-*

或

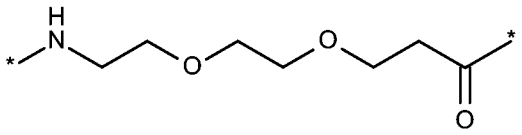
化學式4  ：

當k=1且n=2，該連接子元件可稱為Aeep，其可由以下化學式表示：

化學式5:*-NH-$(CH_2)_2$-O-$(CH_2)_2$-O-$(CH_2)_2$-CO-*

或

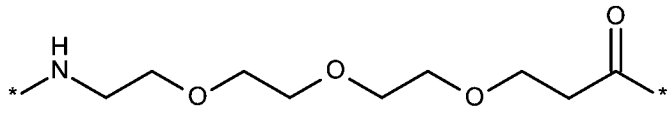
化學式6：



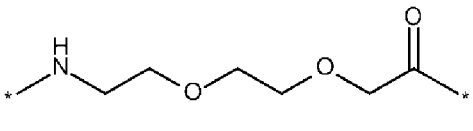
當k=2且n=2，該連接子元件可稱為Aeeep，其可由以下化學式表示：

化學式7：*-NH-$(CH_2)_2$-O-$(CH_2)_2$O-$(CH_2)_2$-O-$(CH_2)_2$-CO-*

或

化學式8：：

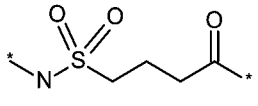
該可選的連接子(L_P)可包含具有以下化學式之8-胺基-3,6-二氧雜-辛酸(OEG)基團

化學式8a：

該可選的連接子(L_P)可包含一個磺醯胺-C4部分。磺醯胺-C4基團為連接至4-丁醯基之磺醯胺基團，且具有以下化學式：

化學式9：*-NH-$S(O)_2$-CH_2-CH_2-CH_2-CO-*

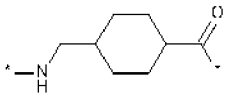
或

化學式10：

該可選的連接子L_P可包括Trx。Trx也被稱為傳明酸（反式-4-(胺基甲基)環己甲酸），且具有以下化學式：

化學式11：*-NH-CH_2-(C_6H_{10})-CO-*

或

化學式12：

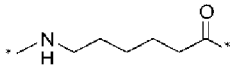
該連接子L_P可包含ε-離胺酸(ε-Lys)。

該連接子L_P可包含離胺酸(Lys)。

該連接子L_P可包含Ahx。Ahx也被稱為胺基己酸（6-胺基己酸），且定義為：

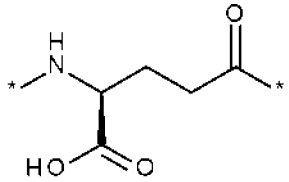
化學式13：*-NH-$(CH_2)_5$-CO-*

或

化學式14：

該連接子L_P可包含一個Glu二基，諸如

化學式15：

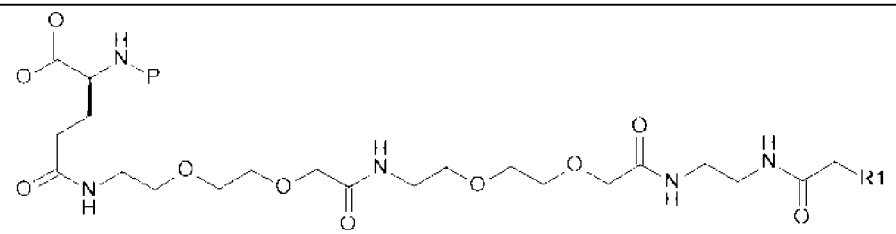
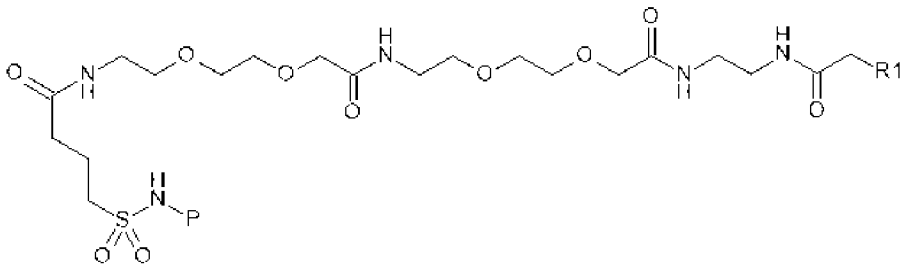


其中該Glu雙自由基可被包含p次，p為1-3範圍內的整數。

【0241】 化學式15也可稱為γ-Glu（或簡稱γGlu），因為它是胺基酸麩胺酸的γ羧基，於此用於連接至離胺酸的ε-胺基。如上所說明，另一個連接子元件可為(例如)另一個Glu殘基或Ado分子。Glu的胺基Glu的胺基轉而與延長部分的羧基或與例如Ado分子的羧基(若存在時)或與例如另一個Glu的γ-羧基(若存在時)形成醯胺鍵。

【0242】 或者，本文所揭露之ISVD多肽衍生物可包含連接子L_P，其係選自下表2中所繪示之任一者。R1表示延長部分所接附之ISVD多肽的殘基，而P表示延長子。

表2：(可選的)延長部分連接子(“L_P”)之實例

連接子(L_P) ID	*結構*	*使用的衍生化合物#*
L_P1		化合物#4、#5、#24、#26、#28、#30、#32、#34、#36、#38、#40、#58、#59、#60、#61、#62、#63、#65、#66、#67、#6、#7、#8、#9、#10、#11、#12、#13、#14、#15、#16、#17、#18、#19、#20、#21、#22
L_P2		化合物#68
L_P3	“R1” - GGGGSGGGGS (SEQ ID NO:25) - “P” 或 “P” - GGGGSGGGGS (SEQ ID NO:25) - “R1”	化合物#70、#72
L_P4	“R1” - GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO:26) – “P” 或 “P” - GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO:26) – “R1”	化合物#71、#73

【0243】 根據本文的揭示內容，熟習該項技藝者將能夠視情況在一些有限的常規實驗後判定用於本文所揭露之特定ISVD多肽衍生物的最佳L_P及L_{1-2}連接子。例如，連接子最好是能夠讓ISVD多肽中的每個ISVD（諸如V_HH片段）與其靶標結合。同樣地，根據本文的揭示內容，熟習該項技藝者將能夠視情況在一些有限的常規實驗後判定用於本文所揭露之特定ISVD多肽衍生物的最佳連接子。

【0244】 在ISVD或ISVD多肽主鏈上發生連接的胺基酸殘基在本文中係稱為R1。在連接超過一個延長部分的情況下，其他的連接點可稱為R2、R3等等。

【0245】 延長部分可接附至該ISVD多肽主鏈之第一ISVD部分中的半胱胺酸或離胺酸殘基。

【0246】 延長部分可接附至該ISVD多肽主鏈之第二ISVD部分中的半胱胺酸或離胺酸殘基。

【0247】 延長部分可接附至該ISVD多肽主鏈之可選的連接子(L_{1-2})部分中的半胱胺酸或離胺酸殘基。

【0248】 該延長部分可以共價方式接附至該ISVD多肽主鏈之離胺酸殘基。該延長部分可經由該延長部分之羧酸基團與該離胺酸殘基之ε胺基之間所形成的醯胺鍵來附接。

【0249】 該延長部分可以共價方式接附至該ISVD多肽主鏈之半胱胺酸殘基。該延長部分可經由該延長部分與該主鏈之半胱胺酸殘基的硫原子之間所形成的硫醚來附接。

【0250】 因此，在一些實施例中本文所揭露之化合物可包含一、二或三個離胺酸或半胱胺酸殘基以及一、二或三個延長部分，其中各延長部分係接附至單個離胺酸或半胱胺酸殘基的側鏈。

【0251】 當經由半胱胺酸殘基發生連接時，該半胱胺酸較佳為游離半胱胺酸。

【0252】 在一些實施例中，游離半胱胺酸係藉由重組DNA技術導入，可作為連接一或多個C16-、C17-或C18二酸γ-Glu 2xOEG脂肪酸分子的共軛位點。

【0253】 在一較佳實施例中，視情況藉由重組DNA技術導入的游離半胱胺酸係可作為連接一、二或三個C18二酸γ-Glu 2xOEG脂肪酸部分的共軛位點

(IUPAC名稱 S{β-AA#}-[2-[2-[[2-[2-[2-[[2-[2-[2-[[(4S)-4-羧基-4-(17-羧基十七醯基-胺基)丁醯基]胺基]乙氧基]乙氧基]乙醯基]胺基]乙氧基]乙氧基]乙醯基]胺基]-乙胺基]-2-側氧基乙基], AA#胺基酸附接)。

【0254】 在另一較佳實施例中，視情況藉由重組DNA技術導入的游離半胱胺酸係可作為連接一、二或三個C17二酸γ-Glu 2xOEG脂肪酸部分的共軛位點

(IUPAC名稱 S{β-AA#}-[2-[2-[[2-[2-[2-[[2-[2-[2-[[(4S)-4-羧基-4-(16-羧基十七醯基-胺基)丁醯基]胺基]乙氧基]乙氧基]乙醯基]胺基]乙氧基]乙氧基]乙醯基]胺基]-乙胺基]-2-側氧基乙基], AA#胺基酸附接)。

【0255】 在一最佳實施例中，視情況藉由重組DNA技術導入的游離半胱胺酸係可作為連接一、二或三個C16二酸γ-Glu 2xOEG脂肪酸部分的共軛位點

(IUPAC名稱 S{β-AA#}-[2-[2-[[2-[2-[2-[[2-[2-[2-[[(4S)-4-羧基-4-(15-羧基十五醯基-胺基)丁醯基]胺基]乙氧基]乙氧基]乙醯基]胺基]乙氧基]乙氧基]乙醯基]胺基]-乙胺基]-2-側氧基乙基], AA#胺基酸附接)。

【0256】 如本文所用之術語「標準層析術」係涵蓋標準層析方法，諸如蛋白質A、陽離子交換、陰離子交換、疏水性交互作用及羥磷石灰層析法。

【0257】 如本文所用之術語「表面暴露胺基酸殘基」係指其側鏈可與溶劑分子（一般來說最可能是水分子）接觸的胺基酸殘基。然而，側鏈不一定要完全與溶劑分子接觸，當甚至部分的側鏈與溶劑分子接觸時，該胺基酸殘基係被定義為「位於表面的胺基酸」。位於多肽表面的胺基酸殘基亦可包括位於靠近ISVD表面的胺基酸殘基，藉以與其他胺基酸殘基有相互的電荷影響，且側鏈甚至部分與

溶劑分子接觸。所屬技術領域中具有通常知識者係可藉由(例如)使用市售或開放供大眾取得的軟體進行同源建模或機器學習，製備多肽或抗體的同源模型或機器學習三維分子模型。或者，也可以使用諸如用於三維分子模型生成之X射線晶體學的方法來生成三維分子模型。可能暴露在表面的胺基酸殘基係可使用(例如)一抗體的三維分子模型座標且使用諸如MOE (Chemical Computing Group)或Bioluminate (Schrödinger)之電腦程式來確定。表面暴露位點係可使用本技術領域中已知的演算法來確定（例如，Lee及Richards (1971) J. Mol. Biol. 55:379-400; Connolly, J. Appl. Cryst. (1983) 16:548-558)。表面可暴露的位點係可使用適於蛋白質建模及分析從該抗體中獲得之三維結構資訊的軟體來確定。可用於這類目的之軟體包括(例如) MOE (Chemical Computing Group) or Bioluminate (Schrödinger)。使用探針半徑為1.4 Å的水探針計算溶劑可及表面（以$Å^2$為單位）的面積。此外，使用個人電腦軟體來確定表面暴露區域及面積的方法係已被Pacios (Pacios, Comput. Chem. 18(4):377-386 (1994); J. Mol. Model. 1:46-53 (1995))描述。根據這類資訊，可選擇出位於抗體表面上與溶劑接觸的適當胺基酸殘基。

醫藥組成物

【0258】 本文所揭露之V_HH多肽衍生物係可製備於醫藥組成物中。在一些實施例中，這類組成物包含至少一醫藥學上可接受之賦形劑。

【0259】 本文所用之術語「賦形劑」廣泛地意指除了活性治療成分(API)以外的任何組分。該賦形劑可用於多種目的，例如作為載體、載劑、填充物、黏合劑、潤滑劑、流動劑、崩解劑、流動控制劑、結晶抑制劑、增溶劑、穩定劑、著色劑、調味劑、界面活性劑、乳化劑、輸送劑、水溶助劑或其組合，及/或改善投予，及/或活性醫藥成分的吸收。

【0260】 所用的各賦形劑之量可在所屬領域習知之範圍內變化。可用於配製口服劑型之技術及賦形劑係描述於英國皇家醫藥學會之出版部門，美國醫藥

協會及醫藥出版社，Sheskey等人編著《Handbook of Pharmaceutical Excipients》第8版(2017)；以及醫藥出版社，Remington及Allen編著《Remington: the Science and Practice of Pharmacy》第22版(2013)中。

【0261】 在較佳實施例中，該包含有V_HH多肽之組成物進一步包含輸送劑及水溶助劑。

【0262】 較佳的輸送劑為N-(8-(2-羥苯甲醯基)胺基)辛酸(NAC)鹽。

【0263】 在一些實施例中，該傳輸劑為如WO2007/121318中所述之N-(8-(2-羥苯甲醯基)胺基)辛酸之鹽類。在一些實施例中，該輸送劑為N-(8-(2-羥苯甲醯基)胺基)辛酸鈉（在本文中稱作「SNAC」），亦稱為8-(柳醯基胺基)辛酸鈉。

【0264】 該醫藥組成物較佳地包含一或多種水溶助劑。水溶助劑與介面活性劑一樣，包括親水部分及疏水部分，且可形成微胞及自聚集，然而它們能溶解溶質而不發生微胞溶解。在一實施例中，該水溶助劑能夠增加SNAC的溶解度。在一實施例中，水溶助劑為菸鹼醯胺(NAM)。

【0265】 於一實施例中，該組成物為固體組成物。該組成物可為適合口服投予之形式，諸如錠劑(tablet)、小藥囊(sachet)或膠囊(capsule)。在一這類實施例中，該組成物係配製成錠劑。本文所提供之固體組成物可加速溶解，從而能夠快速吸收活性醫藥成分。

投予及劑量

【0266】 本文所揭露之化合物（諸如V_HH多肽衍生物）係可在適合的醫藥組成物中以非經口方式投予，諸如靜脈內投予，諸如肌肉內投予，諸如皮下投予。該化合物可經由非腸道途徑投予且較佳為口服投予(PO)。該化合物可預防性投予。該化合物可治療性投予（按需）。

皮下投予

【0267】 該等化合物藉由皮下投予的遞送劑量可為每天約0.01 mg至1 mg

的化合物，較佳為每天約0.05 mg至5 mg，且更佳為每天、每隔一天、每隔三天、每隔四天、每隔五天、每隔六天或每週一次約0.1 mg至約10 mg，視病狀的嚴重性而定。適合的劑量亦可針對特定化合物基於該化合物之特性(包括其活體內半生期或平均滯留時間及其生物活性)作調整。

【0268】 於一實施例中，本發明係有關一種具有含所述組成物之內容物的注射裝置。

口服(PO)投予

【0269】 該等化合物藉由PO遞送的劑量可為每天、每隔一天、每隔三天約1 mg至約300 mg的化合物，視病狀的嚴重性而定。適合的劑量亦可針對特定化合物基於該化合物之特性(包括其活體內半生期或平均滯留時間及其生物活性)作調整。

【0270】 可投予含有於本文中揭露化合物的組成物以用於預防性治療及/或於一些實施例中治療性治療。於治療性應用中，組成物係以足以治癒、減輕或部分地停止疾病(諸如任何出血病症，如以上描述的)及其併發症的量投予至已患有該疾病的個體。適用於完成此的量係定義成「治療有效量」。如所屬技術領域中具有通常知識者會瞭解的，有效於此目的的量會取決於疾病或損傷之嚴重性以及該個體之重量及一般狀態。

【0271】 實施例

1. 一種促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物，包含：

一第一ISVD (ISVD1)，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二ISVD (ISVD2)，其能夠與因子X (SEQ ID NO:2)或其活化型結合，

至少一延長部分，

一可選的連接子(L_{1-2})，其連接ISVD1及ISVD2，及

可選的一或更多個延伸子(E)。

2. 如實施例1所述之ISVD多肽衍生物，其具有下式

ISVD1 – ISVD2，或

ISVD1 – ISVD2 – E，或

ISVD2 – ISVD1 – E，或

E - ISVD1 – ISVD2，或

ISVD1 – L1-2 – ISVD2 – E，或

ISVD2 – L1-2 – ISVD1 – E，或

E - ISVD1 – L1-2 – ISVD2。

3. 如實施例1所述之ISVD多肽衍生物，其具有下式（N端至C端）

ISVD1 – ISVD2，或

ISVD2 – ISVD1，或

ISVD1 – ISVD2 – E，或

ISVD2 – ISVD1 – E，或

E - ISVD1 – ISVD2，或

E – ISVD2 – ISVD1，或

ISVD1 – L1-2 – ISVD2，或

ISVD2 – L1-2 – ISVD1，或

ISVD1 – L1-2 – ISVD2 – E，或

ISVD2 – L1-2 – ISVD1 – E，或

E - ISVD1 – L1-2 – ISVD2，或

E – ISVD2 – L1-2 – ISVD1。

4. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該至少一延長部分係接附至一或多個表面暴露殘基。

5. 如前述實施例中任一例所述之ISVD多肽衍生物，其具有下式（N端至C端）

ISVD2 – L_{1-2} – ISVD1 – E

其中一或多個延長部分係與一或多個表面暴露殘基接附。

6. 如前述實施例中任一例所述之ISVD多肽衍生物，其具有下式（N端至C端）

ISVD2 – L_{1-2} – ISVD1 – E

其中兩個延長部分係與E上的一或多個表面暴露殘基接附。

7. 如前述實施例中任一例所述之ISVD多肽衍生物，其具有下式（N端至C端）

ISVD2 – L_{1-2} – ISVD1 – E

其進一步包括

一第一延長部分及一第二延長部分係分別與E之中的一第一表面暴露殘基及一第二表面暴露殘基接附。

8. 如前述實施例中任一例所述之ISVD多肽衍生物，其中L_{1-2}包含「40%至60%」至「60%至40%」比例的疏水性胺基酸與親水性胺基酸。

9. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）中之至少一者的因子IX (SEQ ID NO:1)或其活化型上的表位。

10. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）中之至少4、5、6、7或8個的因子IX (SEQ ID NO:1)或其活化型上的表位。

11. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）的因子IX (SEQ ID NO:1)或其活化型上的表位。

12. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第二ISVD係能夠結

合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）中之至少一者的因子X (SEQ ID NO:2)上的表位。

13. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177及L178（連續編號）的因子X (SEQ ID NO:2)上的表位。

14. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）中之至少一者的因子X (SEQ ID NO:2)上的表位。

15. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）中之至少3、4、5或6個的因子X (SEQ ID NO:2)上的表位。

16. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）的因子X (SEQ ID NO:2)上的表位。

17. 如前述實施例中任一例所述之ISVD多肽衍生物，其中

該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）中之至少一者的因子IX (SEQ ID NO:1)或其活化型上的表位，且

該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177及L178（連續編號）中之至少一者的因子X (SEQ ID NO:2)上的表位。

18. 如前述實施例中任一例所述之ISVD多肽衍生物，其中

該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）的因子IX (SEQ ID NO:1)或其活化型上的表位，且

該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177、L178及D179（連續編號）的因子X (SEQ ID NO:2)上的表位。

19. 如實施例1至9中任一例所述之ISVD多肽衍生物，其中該第一ISVD係包含一包含有胺基酸殘基F29、N30、Y32、T54、D99、R100、S101、F102、L103、F104、Q106、A107及N113 (SEQ ID NO:35)之互補位，且

其中該第二ISVD係包含一包含有以下胺基酸殘基之互補位

a) D32、A33、M34、G35、Y37、L47、V48、A49、G50、I51、M52、N57、T58、N59、Y60、T61、K97、V99、R101及P102 (SEQ ID NO:27)，或

b) A33、M34、G35、W47、V48、A49、A50、I51、S52、S57、T58、N59、Y60、A61、A97、A98、D99、G105、L107及Y109 (SEQ ID NO:734)（連續編號）。

20. 實施例1至9中任一例所述之ISVD多肽衍生物，其中所述第一ISVD包含：

1)

CDR1：IYTMS (SEQ ID NO:172)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:173)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:174)，其視情況包含一、二或三個胺基酸取代（Kabat定義）。

21. 如實施例1至9中任一例所述之ISVD多肽衍生物，其中所述第二ISVD包含：

(A)

CDR1：RYAMG (SEQ ID NO:168)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:169)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:170)，其視情況包含一、二或三個胺

基酸取代；或

(B)

CDR1：RLAMG (SEQ ID NO:128)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:129)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:130)，其視情況包含一、二或三個胺基酸取代

（Kabat定義）。

22. 如實施例1至9中任一例所述之ISVD多肽衍生物，其中所述第一ISVD包含：

1)

CDR1：IYTMS (SEQ ID NO:172)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:173)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:174)，其視情況包含一、二或三個胺基酸取代；或

且其中所述第二ISVD包含：

(A)

CDR1：RYAMG (SEQ ID NO:168)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:169)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:170)，其視情況包含一、二或三個胺基酸取代；或

(B)

CDR1：RLAMG (SEQ ID NO:128)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:129)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:130)，其視情況包含一、二或三個胺基酸取代

（Kabat編號）。

23. 如實施例20至22中任一例所述之ISVD多肽衍生物，其中所述取代為保守性取代。

24. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述第一ISVD的序列係分別與SEQ ID NO:107、115、123、131、155或171所識別的序列至少90、91、92、93、94、95、96、97、98或99%一致。

25. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述第二ISVD的序列係分別與SEQ ID NO:103、111、119、127、151或167所識別的序列至少90、91、92、93、94、95、96、97、98或99%一致。

26. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述第一ISVD的序列係分別與SEQ ID NO:107、115、123、131、155或171所識別的序列至少90、91、92、93、94、95、96、97、98或99%一致，且其中所述第二ISVD的序列係分別與SEQ ID NO:103、111、119、127、151或167所識別的序列至少90、91、92、93、94、95、96、97、98或99%一致。

27. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述第一ISVD包含以下序列：

V_HH-2.20 (SEQ ID NO:171)，

V_HH-2.18 (SEQ ID NO:155)，

V_HH-2.15 (SEQ ID NO:131)，

V_HH-2.13 (SEQ ID NO:115)，

V_HH-2.14 (SEQ ID NO:123)，或

V_HH-2.12 (SEQ ID NO:107)。

28. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述第二ISVD包含以下序列：

V_HH-1.20 (SEQ ID NO:167)，

V_HH-1.18 (SEQ ID NO:151)，

V_HH-1.15 (SEQ ID NO:127)，

V_HH-1.13 (SEQ ID NO:111)，

V_HH-1.14 (SEQ ID NO:119)，或

V_HH-1.12 (SEQ ID NO:103)。

29. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述第一ISVD包含以下序列：

V_HH-2.20 (SEQ ID NO:171)，

V_HH-2.18 (SEQ ID NO:155)，

V_HH-2.15 (SEQ ID NO:131)，

V_HH-2.13 (SEQ ID NO:115)，

V_HH-2.14 (SEQ ID NO:123)，或

V_HH-2.12 (SEQ ID NO:107)，

且其中

所述第二ISVD包含以下序列：

V_HH-1.20 (SEQ ID NO:167)，

V_HH-1.18 (SEQ ID NO:151)，

V_HH-1.15 (SEQ ID NO:127)，

V_HH-1.13 (SEQ ID NO:111)，

V_HH-1.14 (SEQ ID NO:119)，或

V_HH-1.12 (SEQ ID NO:103)。

30. 如實施例29所述之ISVD多肽衍生物，其中

所述第一ISVD包含序列V_HH-2.20 (SEQ ID NO:171)，且其中

所述第二ISVD包含序列V_HH-1.20 (SEQ ID NO:167)。

31. 如實施例29所述之ISVD多肽衍生物，其中

所述第一ISVD包含序列V_HH-2.18 (SEQ ID NO:155)，且其中

所述第二ISVD包含序列V_HH-1.18 (SEQ ID NO:151)。

32. 如實施例29所述之ISVD多肽衍生物，其中

所述第一ISVD包含序列V_HH-2.15 (SEQ ID NO:131)，且其中

所述第二ISVD包含序列V_HH-1.15 (SEQ ID NO:127)。

33. 如實施例29所述之ISVD多肽衍生物，其中

所述第一ISVD包含序列V_HH-2.13 (SEQ ID NO:115)，且其中

所述第二ISVD包含序列V_HH-1.13 (SEQ ID NO:111)。

34. 如實施例29所述之ISVD多肽衍生物，其中

所述第一ISVD包含序列V_HH-2.14 (SEQ ID NO:123)，且其中

所述第二ISVD包含序列V_HH-1.14 (SEQ ID NO:119)。

35. 如實施例29所述之ISVD多肽衍生物，其中

所述第一ISVD包含序列V_HH-2.12 (SEQ ID NO:107)，且其中

所述第二ISVD包含序列V_HH-1.12 (SEQ ID NO:103)。

36. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該延長部分係包含選自由以下組成之群的延長子：

a) C16二酸、C17二酸、C18二酸、C19二酸、C20二酸、C21二酸、C22二酸，

b) 四唑，

c) 白蛋白結合肽，諸如RLIEDICLPRWGCLWEDD (SEQ ID NO:737)，

d) FcRn結合肽，諸如QRFCTGHFGGLYPCNG (SEQ ID NO:738)，及

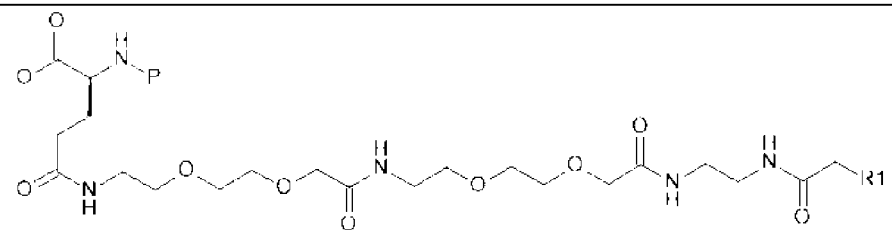
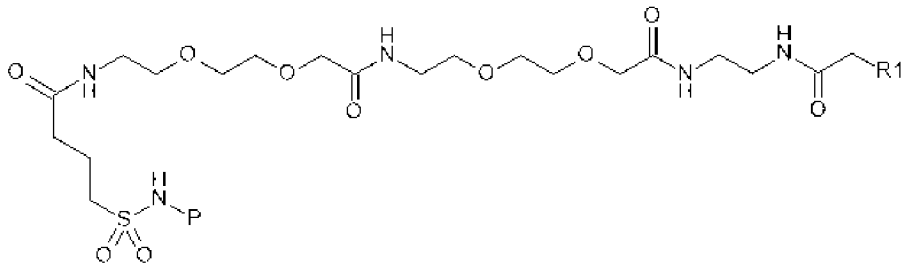
e) Fc結合肽，諸如FNMQQQRRFYEALHDPNLNEEQRNAKIKSIRDDN (SEQ ID NO:739)。

37. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該延長部分係包含選自由以下組成之群的延長子：C16二酸、C17二酸、C18二酸、C19二酸及C20二酸。

38. 如實施例34所述之ISVD多肽衍生物，其中該延長子為C16二酸延長子。

39. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該延長部分係包含一將該延長子接附至ISVD1、L_{1-2}、ISVD2或E的連接子L_P。

40. 如實施例39所述之ISVD多肽衍生物，其中L_P係選自由以下組成之群：

L_P 1	
L_P 2	
L_P 3	“R1” - GGGGSGGGGS (SEQ ID NO:25) – “P” 或 “P” - GGGGSGGGGS (SEQ ID NO:25) – “R1”
L_P 4	“R1” - GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO:26) - “P” 或 “P” - GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO:26) – “R1”

41. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該延長部分係接附至

ISVD2中之位置13及/或ISVD1上之位置12、14、15、42、44、63及/或85（連續編號）中之一或多者。

42. 如實施例1至40中任一例所述之ISVD多肽衍生物，其中該ISVD多肽衍生物包含一C端延伸子，其中該延長部分係接附至所述C端延伸子。

43. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該ISVD多肽衍生物包含一第一延長部分、一第二延長部分及一C端延伸子，其中該第一延長部分及該第二延長部分係接附至該C端延伸子。

44. 如實施例43所述之ISVD多肽衍生物，其中該C端延伸子係接附至ISVD1。

45. 如實施例43所述之ISVD多肽衍生物，其中該C端延伸子係接附至ISVD2。

46. 如前述實施例中任一例所述之ISVD多肽衍生物，其中該等延長部分是相同的。

47. 如前述實施例中任一例所述之ISVD多肽衍生物，其中一或多個延長部分係接附至一表面暴露殘基。

48. 如前述實施例中任一例所述之ISVD多肽衍生物，其中一或多個延長部分係接附至一表面暴露殘基，其中所述表面暴露殘基並不是CDR區中的殘基。

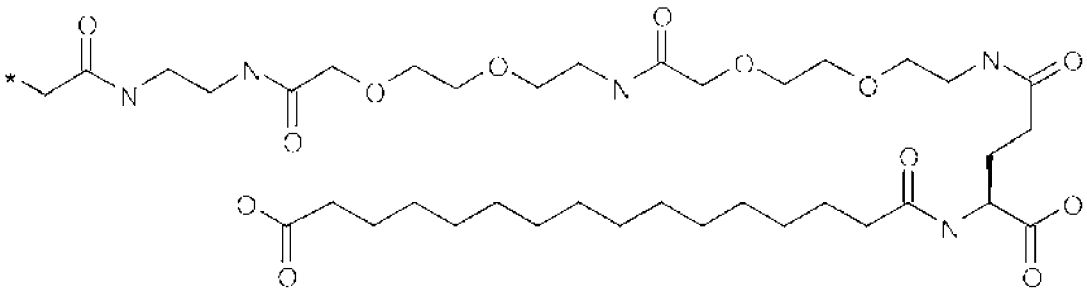
49. 如前述實施例中任一例所述之ISVD多肽衍生物，其中一或多個延長部分係接附至一或多個半胱胺酸或離胺酸殘基。

50. 如實施例43所述之ISVD多肽衍生物，其中

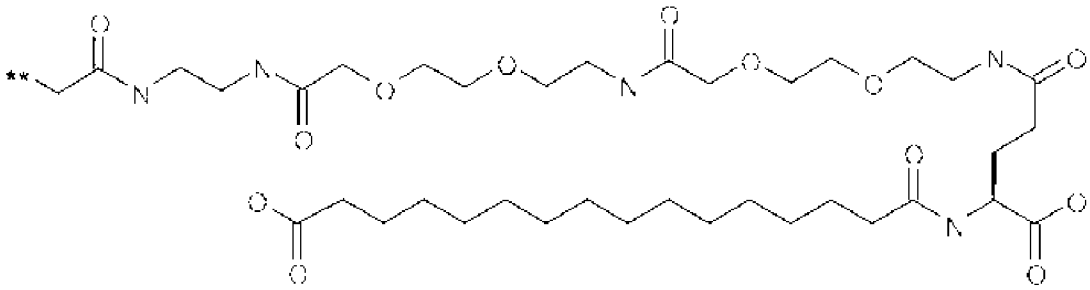
所述ISVD多肽衍生物包含SEQ ID NO:629，且

其中

所述第一延長部分包含以下結構：



且其中所述第二延長部分包含以下結構：



其中「*」(R1)係表示該ISVD多肽之第一延長部分的接附點，且

其中「**」(R2)係表示該ISVD多肽之第二延長部分的接附點，

其中「*」為SEQ ID NO:629之位置257的Cys，且

其中「**」為SEQ ID NO:629之位置259的Cys。

51. 如前述實施例中任一例所述之ISVD多肽衍生物，其中使用等電聚焦所判定之所述多肽的等電位為6.5或更低。

52. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述多肽衍生物為多特異性多肽衍生物，諸如雙或三特異性多肽衍生物。

53. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述多肽衍生物為雙特異性多肽衍生物。

54. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述多肽衍生物的分子量在12-50 kDa的範圍內，諸如12-48 kDa、諸如12-43 kDa、諸如12-37 kDa、諸如12-35 kDa、諸如12-32 kDa、諸如12-27 kDa、諸如12-22 kDa、諸如12-

18 kDa、諸如14-50 kDa、諸如14-48 kDa、諸如14-43 kDa、諸如14-37 kDa、諸如14-35 kDa、14-32 kDa、諸如14-27 kDa、諸如14-22 kDa、諸如14-18 kDa、諸如22-50 kDa、22-48 kDa、諸如22-43 kDa、諸如22-37 kDa、諸如22-35 kDa、諸如22-32 kDa、諸如 22-31 kDa、諸如22-30 kDa、諸如22-29 kDa、諸如22-28 kDa、諸如22-27 kDa、諸如24-50 kDa, 24-48 kDa、諸如24-43 kDa、諸如24-37 kDa、諸如24-35、諸如24-32 kDa、諸如24-31 kDa、24-30 kDa、24-29 kDa、24-28 kDa、諸如24-27 kDa、諸如36-48 kDa、諸如36-43 kDa、諸如36-37 kDa、諸如28-36 kDa、諸如29-33 kDa、諸如29-30、諸如約29、諸如29、諸如約30、諸如30、諸如30-32 kDa、或諸如31 kDa。

55. 如前述實施例中任一例所述之ISVD多肽衍生物，其不是膜靶向ISVD多肽衍生物。

56. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述延長部分不能夠結合細胞膜的組分，諸如胺基磷脂、諸如磷脂絲胺酸及/或磷脂醯乙醇胺。

57. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述延長部分不能夠結合血小板表面蛋白，諸如GPlb-lX、膠原蛋白伴護蛋白HSP47、ephrin B1、硫醇異構酶蛋白ERP5、造血前驅激酶1-相互作用蛋白55 (HIP-55)、醣蛋白Vl、血小板醣蛋白1b、血小板衍性生長因子受體、血小板內皮聚集受體I、CD31、CD36、MARKS、multimerin、整聯蛋白αIIb/β 3、骨髓細胞上的觸發受體(TREM)樣轉錄體-1 (TLT-1)、整聯蛋白-連接激酶(ILK)、zyxin、膠原蛋白、P-選滯蛋白、因子XIII、P-選滯蛋白醣蛋白配體-1、整聯蛋白α6β1、血小板反應素、溫韋伯氏凝血因子、G6B、CD42b、突觸融合蛋白(syntaxin)節核蛋白2、磷脂醯乙醇胺、纖維蛋白原/纖維蛋白、細絲蛋白(filamin)、口型蛋白、神經鞘脂、CD31、CD36、CD40、CD41、CD42c、CD42、CD49b、CD61、CD62P、CD63、CD69、CD107a、CD107b、CD109、CD154、PECAM-

1及/或ERPS。

58. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述延長部分不能夠結合細胞膜聯結蛋白，諸如醣蛋白、GPIIb/IIIa、β2GP1、TLT-1、選滯蛋白、凝血因子或凝血因子複合體、及/或選滯蛋白。

59. 如前述實施例中任一例所述之ISVD多肽衍生物，其中與沒有所述延長部分的ISVD多肽衍生物相比，在血液中的平均停留時間終末半衰期係延長至少12小時、至少24小時、至少48小時、至少3、4、5或7天。

60. 如前述實施例中任一例所述之ISVD多肽衍生物，其中所述第一及/或第二ISVD為V_HH片段。

61. 如實施例1至59中任一例所述之ISVD多肽衍生物，其中所述第一及/或第二ISVD為V-NAR片段。

62. 如實施例1至60中任一例所述之ISVD多肽衍生物，其中所述ISVD多肽衍生物為V_HH多肽衍生物。

63. 一種促凝血V_HH多肽衍生物，包含：

一第一V_HH，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二V_HH，其能夠與因子X (SEQ ID NO:2)結合，

一連接子(L_{1-2})，其連接所述第一V_HH及所述第二V_HH，及

一C端延伸子(E)，及

一或二個延長部分，

其具有下式（N端至C端）：

「第二V_HH」 – L_{1-2} – 「第一V_HH」 – E

其中

I)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:172)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:173)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:174)，且

所述第二V_HH包含

CDR1：RYAMG (SEQ ID NO:168)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:169)，

CDR3：DDSVGDGYLDY (SEQ ID NO:170)；或

II)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:132)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:133)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:134)，且

所述第二V_HH包含

CDR1：RLAMG (SEQ ID NO:128)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:129)，

CDR3：DDSVGDGYLDY (SEQ ID NO:130)；或

（Kabat定義）。

64. 如實施例63所述之促凝血V_HH多肽衍生物，其中

I)

所述第一V_HH包含序列V_HH-2.20 (SEQ ID NO:171)，且

所述第二V_HH包含序列V_HH-1.20 (SEQ ID NO:167)。

II)

所述第一V_HH包含序列V_HH-2.18 (SEQ ID NO:155)，且

所述第二V_HH包含序列V_HH-1.18 (SEQ ID NO:151)。

III)

所述第一V_HH包含序列V_HH-2.15 (SEQ ID NO:131)，且

所述第二V_HH包含序列V_HH-1.15 (SEQ ID NO:127)。

IV)

所述第一V_HH包含序列V_HH-2.13 (SEQ ID NO:115)，且

所述第二V_HH包含序列V_HH-1.13 (SEQ ID NO:111)。

V)

所述第一V_HH包含序列V_HH-2.14 (SEQ ID NO:123)，且

所述第二V_HH包含序列V_HH-1.14 (SEQ ID NO:119)。

VI)

所述第一V_HH包含序列V_HH-2.12 (SEQ ID NO:107)，且

所述第二V_HH包含序列V_HH-1.12 (SEQ ID NO:103)。

65. 如實施例1至60及62至64中任一例所述之促凝血V_HH多肽衍生物，其中所述V_HH多肽衍生物為雙特異性V_HH多肽衍生物。

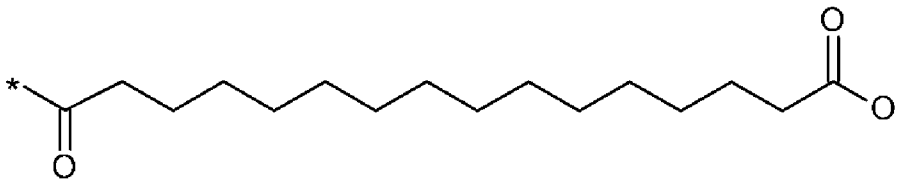
66. 如實施例63至65中任一例所述之促凝血V_HH多肽衍生物，其中該連接子L_{1-2}包含胺基酸殘基GQAPGQ (SEQ ID NO:20)。

67. 如實施例63至65中任一例所述之促凝血V_HH多肽衍生物，其中該延伸子(E)包含胺基酸殘基GQACPC (SEQ ID NO:9)。

68. 如實施例63至67中任一例所述之促凝血V_HH多肽衍生物，其中兩個延長部分係分別接附於E的位置4及6 (SEQ ID NO:9)。

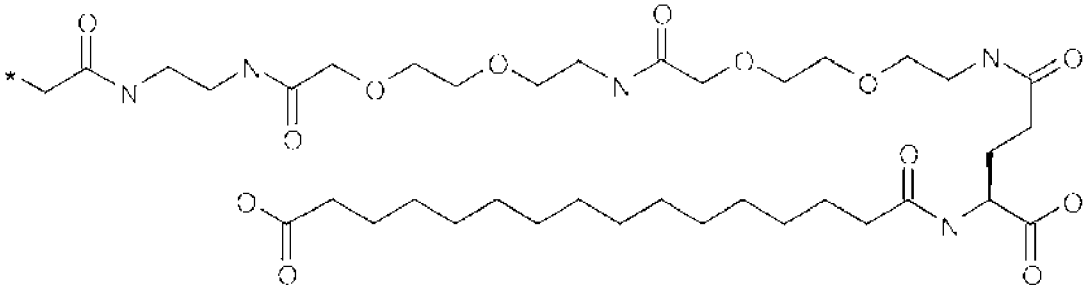
69. 如實施例63至68中任一例所述之促凝血V_HH多肽衍生物，其中所述兩個延長部分是相同的。

70. 如實施例63至69中任一例所述之促凝血V_HH多肽衍生物，其中所述兩個延長部分係包含以下結構：

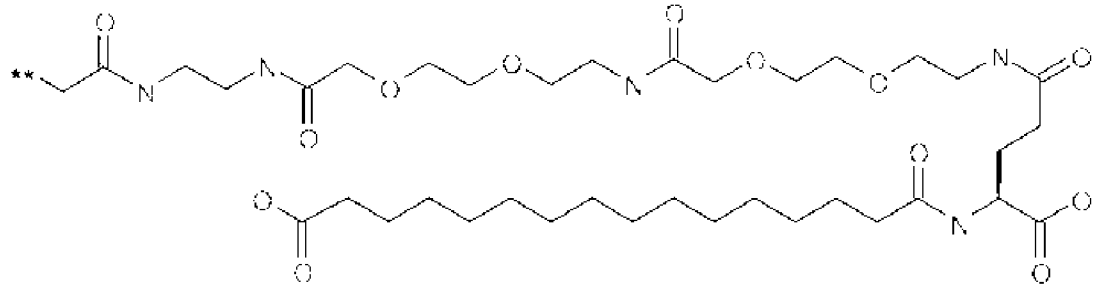


71. 如實施例63至70中任一例所述之促凝血V_HH多肽衍生物，其包含一第一延長部分及一第二延長部分

其中所述第一延長部分包含以下結構：



且其中所述第二延長部分包含以下結構：



其中「*」(R1)係表示該V_HH多肽衍生物之第一延長部分的接附點，且

其中「**」(R2)係表示該V_HH多肽衍生物之第二延長部分的接附點，

其中「*」為SEQ ID NO:629之位置257的Cys，且

其中「**」為SEQ ID NO:629之位置259的Cys。

72. 一種包含有如實施例1至71中任一例所述之ISVD多肽衍生物或V_HH多肽衍生物的醫藥組成物。

73. 一種包含有如實施例1至71中任一例所述之ISVD多肽衍生物或V_HH多肽衍生物的口服投予醫藥組成物。

74. 如實施例72或73所述之醫藥組成物，其中該ISVD多肽衍生物為V_HH多肽衍生物。

75. 如實施例72或73所述之醫藥組成物，其中該ISVD多肽衍生物為V-NAR多肽衍生物。

76. 如實施例72至75中任一例所述之醫藥組成物，其中所述組成物包含N-(8-(2-羥基苯甲醯基)胺基)辛酸鹽。

77. 如實施例76所述之醫藥組成物，其中所述N-(8-(2-羥基苯甲醯基)胺基)辛酸鹽為N-(8-(2-羥基苯甲醯基)胺基)辛酸鈉(SNAC)。

78. 如實施例76至77中任一例所述之醫藥組成物，其進一步包含菸鹼醯胺(NAM)。

79. 如實施例74至78中任一例所述之醫藥組成物，其中所述組成物包含V_HH多肽衍生物、N-(8-(2-羥基苯甲醯基)胺基)辛酸鈉(SNAC)、菸鹼醯胺(NAM)及硬脂酸鎂。

80. 如實施例74至78中任一例所述之醫藥組成物，其中所述組成物包含約7 % w/w的V_HH多肽衍生物、約55 % w/w的N-(8-(2-羥基苯甲醯基)胺基)辛酸鈉(SNAC)、約36 % w/w的菸鹼醯胺(NAM)及約0.5 % w/w的硬脂酸鎂。

81. 如實施例73至80中任一例所述之醫藥組成物，其中所述組成物為液體組成物。

82. 如實施例73至80中任一例所述之醫藥組成物，其中所述組成物為固體組成物。

83. 如實施例82所述之醫藥組成物，其中所述組成物為錠劑、口含錠或膠囊。

84. 一種投予如實施例1至83中任一例所述之ISVD多肽衍生物或V_HH多肽衍生

物或醫藥組成物以用於治療有需要的患者之方法。

85. 如實施例84所述之治療方法，其中該患者為罹患具有或沒有抑制子之A型血友病、或後天性A型血友病的患者。

86. 如實施例84或85所述之治療方法，其中該治療為預防性治療。

87. 如實施例84至86中任一例所述之治療方法，其中該ISVD多肽衍生物、V_HH多肽衍生物或醫藥組成物係口服投予。

88. 如實施例84至86中任一例所述之治療方法，其中該ISVD多肽衍生物、V_HH多肽衍生物或醫藥組成物係皮下注射投予。

89. 如實施例1至83中任一例所述之ISVD多肽衍生物、V_HH多肽衍生物或醫藥組成物，其係供使用於醫藥用途。

90. 如實施例1至83中任一例所述之ISVD多肽衍生物、V_HH多肽衍生物或組成物，其係使用於血友病的治療中，諸如具有或沒有抑制子之A型血友病、或諸如後天性A型血友病。

91. 如實施例1至83中任一例所述之ISVD多肽衍生物、V_HH多肽衍生物或組成物，其係使用於具有或沒有抑制子之A型血友病的治療中。

92. 如實施例89至92中任一例所述之ISVD多肽衍生物、V_HH多肽衍生物或組成物，其中該治療為預防性治療。

93. 如用於實施例89至92中任一例之ISVD多肽衍生物、V_HH多肽衍生物或組成物，其中所述衍生物或組成物係口服投予。

94. 如用於實施例89至92中任一例之ISVD多肽衍生物、V_HH多肽衍生物或組成物，其中所述衍生物或組成物係以皮下注射投予。

95. 一種產生促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物之方法，所述多肽衍生物包含：

一第一ISVD (ISVD1)，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二ISVD (ISVD2)，其能夠與因子X (SEQ ID NO:2)或其活化型結合，

一或多個延長部分，

一可選的連接子(「L_{1-2}」)，其能夠連接ISVD1及ISVD2，及

可選的一或更多個延伸子(「E」)，

該方法包含以下步驟：

a. 將編碼該ISVD多肽或ISVD多肽衍生物之胺基酸殘基的核酸修飾，以降低該ISVD多肽或ISVD多肽衍生物的等電位，

b. 培養宿主細胞以表現編碼該ISVD多肽或ISVD多肽衍生物的核酸，

c. 從宿主細胞培養物收集該ISVD多肽或ISVD多肽衍生物，

d. 使用標準層析法將來自宿主細胞培養物的ISVD多肽或ISVD多肽衍生物純化，及

e. 將一延長部分接附至該ISVD多肽，除非這樣的部分已經存在。

96. 一種產生促凝血免疫球蛋白單可變結構域(ISVD)多肽或ISVD多肽衍生物之方法，所述多肽或多肽衍生物包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合之第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、一延長部分、一連接ISVD1及ISVD2之可選的連接子(「L_{1-2}」)、以及可選的一或更多個延伸子(「E」)，該方法包含以下步驟：

a. 將編碼該ISVD多肽或ISVD多肽衍生物之胺基酸殘基的核酸修飾，以使該ISVD多肽或ISVD多肽衍生物的等電位為6.5或更低，

b. 培養宿主細胞以表現編碼該ISVD多肽或ISVD多肽衍生物的核酸，

c. 從宿主細胞培養物收集該ISVD多肽或ISVD多肽衍生物，

d. 使用標準層析法將來自宿主細胞培養物的ISVD多肽或ISVD多肽衍生物純化，及

e. 將一延長部分接附至該ISVD多肽，除非這樣的部分已經存在。

97. 一種增加促凝血免疫球蛋白單可變結構域(ISVD)多肽或ISVD多肽衍生物之口服生物可利用率的方法，所述多肽或多肽衍生物包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合之第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、一延長部分、一能夠連接ISVD1及ISVD2之可選的連接子(「L_{1-2}」)、以及可選的一或更多個延伸子(「E」)，該方法包含以下步驟：

a. 將編碼該ISVD多肽或ISVD多肽衍生物之胺基酸殘基的核酸修飾，以降低該ISVD多肽或ISVD多肽衍生物的等電位，

b. 培養宿主細胞以表現編碼該ISVD多肽或ISVD多肽衍生物的核酸，

c. 從宿主細胞培養物收集該ISVD多肽或ISVD多肽衍生物，

d. 使用標準層析法將來自宿主細胞培養物的ISVD多肽或ISVD多肽衍生物純化，及

e. 將一延長部分接附至該ISVD多肽，除非這樣的部分已經存在。

98. 一種增加促凝血免疫球蛋白單可變結構域(ISVD)多肽或ISVD多肽衍生物之口服生物可利用率的方法，所述多肽或多肽衍生物包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合之第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、一延長部分、一能夠連接ISVD1及ISVD2之可選的連接子(「L_{1-2}」)、以及可選的一或更多個延伸子(「E」)，該方法包含以下步驟：

a. 將編碼該ISVD多肽或ISVD多肽衍生物之胺基酸殘基的核酸修飾，以使該ISVD多肽或ISVD多肽衍生物的等電位為6.5或更低，

b. 培養宿主細胞以表現編碼該ISVD多肽或ISVD多肽衍生物的核酸，

c. 從宿主細胞培養物收集該ISVD多肽或ISVD多肽衍生物，

d. 使用標準層析法將來自宿主細胞培養物的ISVD多肽或ISVD多肽衍生

物純化，及

e. 將一延長部分接附至該ISVD多肽，除非這樣的部分已經存在。

99. 如實施例95至98中任一例所述之方法，其中該等電點係降低至少0.5 pH單位，諸如至少0.6、0.7、0.8、0.9、1、1.1、1.2、1.3、1.4、1.5、1.6、1.7、1.8、1.9、2、2.1、2.2、2.3、2.4、2.5、2.6、2.7、2.8、2.9、3. 3.1、3.2、3.3、3.4、3.5、3.6、3.7、3.8、3.9或4 pH單位。

100. 如實施例95至99中任一例所述之方法，其中步驟e)不會導致該ISVD多肽或ISVD多肽衍生物的等電點增加。

101. 如實施例95至100中任一例所述之方法，其中該等電點係使用等電聚焦判定。

102. 如實施例95至101中任一例所述之方法，其中該ISVD多肽或ISVD多肽衍生物係由單個核酸所編碼。

103. 如實施例95至101中任一例所述之方法，其中該ISVD多肽或ISVD多肽衍生物為第一及第二ISVD的共軛物，其中所述第一及第二ISVD係由不同的核酸所編碼。

104. 如實施例95至103中任一例所述之方法，其中該ISVD多肽或ISVD多肽衍生物係為V_HH多肽或V_HH多肽衍生物。

105. 一種使用如實施例95至103中任一例所述之方法所產生的ISVD多肽或ISVD多肽衍生物。

106. 一種使用如實施例95至103中任一例所述之方法所可得到的ISVD多肽或ISVD多肽衍生物。

107. 一種使用如實施例95至104中任一例所述之方法所產生的V_HH多肽或V_HH多肽衍生物。

108. 一種能夠結合FIX (SEQ ID NO:1)或其活化型(FIXa)之V_HH片段，

其中

a. 該CDR1序列係由SEQ ID NO:172所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:173所識別，其視情況包含一、二或三個胺基酸取代，

該CDR3序列係由SEQ ID NO:174所識別，其視情況包含一、二或三個胺基酸取代；或

b. 該CDR1序列係由SEQ ID NO:156所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:157所識別，其視情況包含一、二或三個胺基酸取代，

該CDR3序列係由SEQ ID NO:158所識別，其視情況包含一、二或三個胺基酸取代；或

c. 該CDR1序列係由SEQ ID NO:132所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:133所識別，其視情況包含一、二或三個胺基酸取代，

該CDR3序列係由SEQ ID NO:134所識別，其視情況包含一、二或三個胺基酸取代；或

d. 該CDR1序列係由SEQ ID NO:116所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:117所識別，其視情況包含一、二或三個胺基酸取代，

該CDR3序列係由SEQ ID NO:118所識別，其視情況包含一、二或三個胺基酸取代；或

e. 該CDR1序列係由SEQ ID NO:124所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:125所識別，其視情況包含一、二或三個胺基酸取代，

該CDR3序列係由SEQ ID NO:126所識別，其視情況包含一、二或三個胺基酸取代；或

f. 該CDR1序列係由SEQ ID NO:108所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:109所識別，其視情況包含一、二或三個胺基酸取代，

該CDR3序列係由SEQ ID NO:110所識別，其視情況包含一、二或三個胺基酸取代

（Kabat定義）。

109. 一種能夠結合FX (SEQ ID NO:2)之V_HH片段，

其中

a. 該CDR1序列係由SEQ ID NO:152所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:153所識別，其視情況包含一、二或三個胺基

酸取代，

該CDR3序列係由SEQ ID NO:154所識別，其視情況包含一、二或三個胺基酸取代；或

b. 該CDR1序列係由SEQ ID NO:128所識別，其視情況包含一或二個胺基酸取代，

該CDR2序列係由SEQ ID NO:129所識別，其視情況包含一、二或三個胺基酸取代，

該CDR3序列係由SEQ ID NO:130所識別，其視情況包含一、二或三個胺基酸取代。

110. 如實施例108或109所述之V_HH片段，其中所述取代為保守性取代。

111. 如實施例108至110中任一例所述之V_HH片段，其中所述V_HH片段係為供使用於製造能夠與FIX (SEQ ID NO:1)或其活化型(FIXa)結合、且能夠與FX (SEQ ID NO:2)或其活化型(FXa)結合之V_HH多肽或V_HH多肽衍生物的中間物。

112. 如實施例108至110中任一例所述之V_HH片段，其中所述V_HH多肽係為供使用於製造ISVD多肽衍生物的中間物。

113. 如實施例109或110所述之ISVD或V_HH，其中與WO2019/096874所述之任一抗FX ISVD化合物相比下，在使FX更易於被FIXa進行蛋白水解方面的活性係得到改善。

114. 一種促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物，包含：

一第一ISVD (ISVD1)，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二ISVD (ISVD2)，其能夠與因子X (SEQ ID NO:2)或其活化型結合，

至少一延長部分，

一可選的連接子(L_{1-2})，其連接ISVD1及ISVD2，及

可選的一或更多個延伸子(E)，

其中所述ISVD多肽係選自由 SEQ ID NO:615-691、734或735組成之群。

115. 一種V_HH片段，其包含SEQ ID NO:27-614中之任一者的序列。

116. 一種V_HH多肽衍生物，其包含SEQ ID NO: 615-691或734-735中之任一者的序列。

117. 一種促凝血免疫球蛋白單可變結構域(ISVD)多肽衍生物，包含：

一第一ISVD (ISVD1)，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二ISVD (ISVD2)，其能夠與因子X (SEQ ID NO:2)或其活化型結合，

至少一延長部分，

一可選的連接子(L_{1-2})，其連接ISVD1及ISVD2，及

可選的一或更多個延伸子(E)。

118. 如實施例117所述之ISVD多肽衍生物，其具有下式（N端至C端）

ISVD2 – L_{1-2} – ISVD1 – E

其中一或多個延長部分係與一或多個表面暴露殘基接附。

119. 如實施例118或119所述之ISVD多肽衍生物，其具有下式（N端至C端）

ISVD2 – L_{1-2} – ISVD1 – E

其中兩個延長部分係與E上的一或多個表面暴露殘基接附，且

其中所述衍生物的分子量係於20-35 kDa的範圍內。

120. 如實施例117至119中任一例所述之ISVD多肽衍生物，其中

該第一ISVD係能夠結合至包含有胺基酸殘基E224、T225、G226、V250、I251、R252、I253、P255、H257及N260（連續編號）的因子IX (SEQ ID NO:1)或其活化型上的表位，且

該第二ISVD係能夠結合至包含有胺基酸殘基N173、P174、F175、L177及L178（連續編號）的因子X (SEQ ID NO:2)或其活化型上的表位。

121. 如實施例117至119中任一例所述之ISVD多肽衍生物，其中所述第一ISVD包含：

1)

CDR1：IYTMS (SEQ ID NO:172)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:173)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:174)，其視情況包含一、二或三個胺基酸取代；或

2)

CDR1：IYTMS (SEQ ID NO:156)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:157)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:158)，其視情況包含一、二或三個胺基酸取代；或

3)

CDR1：IYTMS (SEQ ID NO:132)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:133)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:134)，其視情況包含一、二或三個胺基酸取代；或

4)

CDR1：IYTMS (SEQ ID NO:116)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:117)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:118)，其視情況包含一、二或三個胺基酸取代；或

5)

CDR1：IYTMS (SEQ ID NO:124)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:125)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:126)，其視情況包含一、二或三個胺基酸取代；或

6)

CDR1：IYTMS (SEQ ID NO:108)，其視情況包含一或二個胺基酸取代，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:109)，其視情況包含一、二或三個胺基酸取代，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:110)，其視情況包含一、二或三個胺基酸取代；

且其中所述第二ISVD包含：

(A)

CDR1：RYAMG (SEQ ID NO:168)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:169)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:170)，其視情況包含一、二或三個胺基酸取代；或

(B)

CDR1：RYAMG (SEQ ID NO:152)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:153)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:154)，其視情況包含一、二或三個胺基酸取代；或

(C)

CDR1：RLAMG (SEQ ID NO:128)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:129)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:130)，其視情況包含一、二或三個胺基酸取代；或

(D)

CDR1：RLAMG (SEQ ID NO:112)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:113)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:114)，其視情況包含一、二或三個胺基酸取代；或

(E)

CDR1：RLAMG (SEQ ID NO:120)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:121)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:122)，其視情況包含一、二或三個胺基酸取代；或

(F)

CDR1：RLAMG (SEQ ID NO:104)，其視情況包含一或二個胺基酸取代，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:105)，其視情況包含一、二或三個胺基酸取代，

CDR3：DDSVGDGYLDY (SEQ ID NO:106)，其視情況包含一、二或三個胺基酸取代

（Kabat定義）。

122. 如實施例121所述之ISVD多肽衍生物，其中所述取代為保守性取代。

123. 如實施例117至122中任一例所述之ISVD多肽衍生物，其中所述第一ISVD包含以下序列：

V_HH-2.20 (SEQ ID NO:171)，

V_HH-2.18 (SEQ ID NO:155)，

V_HH-2.15 (SEQ ID NO:131)，

V_HH-2.13 (SEQ ID NO:115)，

V_HH-2.14 (SEQ ID NO:123)，或

V_HH-2.12 (SEQ ID NO:107)，

且其中

所述第二ISVD包含以下序列：

V_HH-1.20 (SEQ ID NO:167)，

V_HH-1.18 (SEQ ID NO:151)，

V_HH-1.15 (SEQ ID NO:127)，

V_HH-1.13 (SEQ ID NO:111)，

V_HH-1.14 (SEQ ID NO:119)，或

V_HH-1.12 (SEQ ID NO:103)。

124. 一種促凝血V_HH多肽衍生物，包含：

一第一V_HH，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二V_HH，其能夠與因子X (SEQ ID NO:2)或其活化型結合，

一連接子(L_{1-2})，其連接所述第一V_HH及所述第二V_HH，及

一C端延伸子(E)，及

一或二個延長部分，

其具有下式（N端至C端）：

「第二V_HH」 – L_{1-2} – 「第一V_HH」 – E

其中

I)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:172)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:173)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:174)，且

所述第二V_HH包含

CDR1：RYAMG (SEQ ID NO:168)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:169)，

CDR3：DDSVGDGYLDY (SEQ ID NO:170)；或

II)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:156)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:157)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:158)，且

所述第二V_HH包含

CDR1：RYAMG (SEQ ID NO:152)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:153)，

CDR3：DDSVGDGYLDY (SEQ ID NO:154)；或

III)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:132)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:133)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:134)，且

所述第二V_HH包含

CDR1：RLAMG (SEQ ID NO:128)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:129)，

CDR3：DDSVGDGYLDY (SEQ ID NO:130)；或

IV)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:116)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:117)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:118)，且

所述第二V_HH包含

CDR1：RLAMG (SEQ ID NO:112)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:113)，

CDR3：DDSVGDGYLDY (SEQ ID NO:114)；或

V)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:124)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:125)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:126)，且

所述第二V_HH包含

CDR1：RLAMG (SEQ ID NO:120)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:121)，

CDR3：DDSVGDGYLDY (SEQ ID NO:122)；或

VI)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:108)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:109)，

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:110)，且

所述第二V_HH包含

CDR1：RLAMG (SEQ ID NO:104)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:105)，

CDR3：DDSVGDGYLDY (SEQ ID NO:106)

（Kabat定義）。

125. 一種包含有如實施例117至124中任一例所述之ISVD多肽衍生物或V_HH多肽衍生物的醫藥組成物。

126. 如實施例125所述之醫藥組成物，其中所述組成物包含N-(8-(2-羥基苯甲醯基)胺基)辛酸鹽。

127. 如實施例126所述之醫藥組成物，其中所述N-(8-(2-羥基苯甲醯基)胺基)辛酸鹽為N-(8-(2-羥基苯甲醯基)胺基)辛酸鈉(SNAC)。

128. 如實施例125至127中任一例所述之醫藥組成物，其進一步包含菸鹼醯胺

(NAM)。

129. 如實施例125至128中任一例所述之醫藥組成物，其中所述組成物為固體組成物。

130. 如實施例117至129中任一例所述之ISVD多肽衍生物、V_HH多肽衍生物或組成物，其係使用於血友病的治療中，諸如具有或沒有抑制子之A型血友病、或諸如後天性A型血友病。

131. 如用於實施例130之ISVD多肽衍生物、V_HH多肽衍生物或組成物，其中所述多肽衍生物或組成物係口服投予。

132. 一種增加促凝血免疫球蛋白單可變結構域(ISVD)多肽或ISVD多肽衍生物之口服生物可利用率的方法，所述多肽或多肽衍生物包含一能夠與因子IX (SEQ ID NO:1)或其活化型結合之第一ISVD (ISVD1)、一能夠與因子X (SEQ ID NO:2)或其活化型結合之第二ISVD (ISVD2)、一延長部分、一能夠連接ISVD1及ISVD2之可選的連接子(「L_{1-2}」)、以及可選的一或更多個延伸子(「E」)，該方法包含以下步驟：

a. 將編碼該ISVD多肽或ISVD多肽衍生物之胺基酸殘基的核酸修飾，以使其等電位降低，

b. 培養宿主細胞以表現編碼該ISVD多肽或ISVD多肽衍生物的核酸，

c. 從宿主細胞培養物收集該ISVD多肽或ISVD多肽衍生物，

d. 使用標準層析法將來自宿主細胞培養物的ISVD多肽或ISVD多肽衍生物純化，及

e. 將一延長部分接附至該ISVD多肽，除非這樣的部分已經存在。

【0272】 於一較佳實施例中，ISVD多肽衍生物包含一個以N端到C端方向(N到C端)連接至抗FIX(a) V_HH ISVD的抗FX ISVD。

【0273】 於一這類的較佳實施例中，ISVD多肽衍生物包含一個以N端到C端方向(N到C端)連接至抗FIX(a) V_HH片段的抗FX V_HH片段，見(例如)圖2a、2b及3a-f。

【0274】 於一較佳實施例中，該能夠結合FIX(a)之ISVD或V_HH片段係優先結合至活化的FIX (FIXa)。

【0275】 於一較佳實施例中，該能夠結合FX之ISVD或V_HH片段係結合FX酶原，即尚未活化的FX。

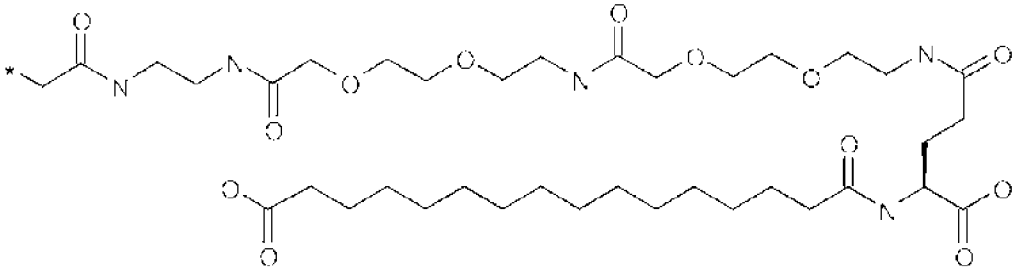
【0276】 於一實施例中，本發明之ISVD多肽(衍生物)或V_HH多肽(衍生物)係能夠刺激FIXa對於FX的酶活性。在一這類實施例中，所述ISVD多肽(衍生物)或V_HH多肽(衍生物)之刺激特性係衍生自該結合FIX(a)之ISVD或V_HH。

【0277】 圖5a及5b顯示了較佳的抗FIX(a)及抗FX ISVD (V_HH片段)序列的序列比對，其中CDR序列係以黑體及下劃線標出。

【0278】 本發明涵蓋了本文所揭露之ISVD或V_HH片段的取代變異體，其在本文所揭露之個別序列中可包含1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20個胺基酸取代及/或缺失及/或插入。於一些實施例中，所述取代及/或缺失及/或插入係於本文所揭露之一或多個CDR序列之中。於一些實施例中，該等CDR序列中之一或多者係受到胺基酸取代。於一些實施例中，個別的CDR序列可各包含0、1、2或3個胺基酸取代。例如，在SEQ ID NO:559的V_HH片段中，CDR1可不包含取代，CDR2可包含3個取代，而CDR3可包含1個取代。取代變異體較佳地係涉及用相同數目的胺基酸替代一或多個胺基酸。於一些實施例中，該取代為保守性取代。

【0279】 於一實施例中，該ISVD多肽衍生物（諸如V_HH多肽衍生物）係包含至少一延長部分，所述延長部分不能夠結合細胞膜的組分，諸如胺基磷脂、諸如磷脂絲胺酸及/或磷脂醯乙醇胺。

【0280】 在一實施例中，該延長部分包含以下結構：



其中「*」係表示該V_HH多肽之接附點。

【0281】 於一實施例中，該ISVD多肽衍生物（諸如V_HH多肽衍生物）係包含一延長部分，所述延長部分不能夠結合血小板表面蛋白，諸如GPlb-lX、膠原蛋白伴護蛋白HSP47、ephrin B1、硫醇異構酶蛋白ERP5、造血前驅激酶1-相互作用蛋白55 (HIP-55)、醣蛋白Vl、血小板醣蛋白1b、血小板衍性生長因子受體、血小板內皮聚集受體I、CD31、CD36、MARKS、multimerin、整聯蛋白α llb/β 3、骨髓細胞上的觸發受體(TREM)樣轉錄體-1 (TLT-1)、整聯蛋白-連接激酶(ILK)、zyxin、膠原蛋白、P-選滯蛋白、因子XIII、P-選滯蛋白醣蛋白配體-1、整聯蛋白α6β1、血小板反應素、溫韋伯氏凝血因子、G6B、CD42b、突觸融合蛋白(syntaxin)節核蛋白2、磷脂醯乙醇胺、纖維蛋白原/纖維蛋白、細絲蛋白(filamin)、口型蛋白、神經鞘脂、CD31、CD36、CD40、CD41、CD42c、CD42、CD49b、CD61、CD62P、CD63、CD69、CD107a、CD107b、CD109、CD154、PECAM-1及/或ERPS。

【0282】 於一實施例中，該ISVD多肽衍生物（諸如V_HH多肽衍生物）係包含一延長部分，所述延長部分不能夠結合細胞膜聯結蛋白，諸如醣蛋白、GPIIb/IIIa、β2GP1、TLT-1、選滯蛋白、凝血因子或凝血因子複合體、及/或選滯蛋白。

【0283】 於一實施例中，本發明之ISVD多肽衍生物（諸如V_HH多肽衍生物）於所述多肽衍生物以臨床相關劑量使用於A型血友病的治療中，不會干擾FVIII（諸如向患有A型血友病之患者所投予的重組FVIII）的作用。

【0284】 儘管本發明之某些特徵在本文中已說明及描述，但本領域普通技術人員現將聯想到許多修飾、取代、變化及等效物。因此，應當理解，所附申請專利範圍旨在涵蓋落入本發明真正精神之所有此類修飾及改變。

實例

【0285】 縮寫列表

AIEX: 陰離子交換

CC: Pearson相關係數

CDR: 互補決定區

CHO: 中國倉鼠卵巢

CMK: 氯甲基酮

EC_{50}: 半最大有效濃度

EDTA： 乙二胺四乙酸

ELISA: 酵素聯結免疫吸附分析法

FACS: 螢光流式細胞分選儀

FIX: 凝血因子IX

FIXa: 凝血因子IXa

FX: 凝血因子X

FXa: 凝血因子Xa

FR: 構架區

GS: 麩醯胺合成酶

HA: A型血友病

HB: B型血友病

ISVD: 免疫球蛋白單可變結構域

IV: 靜脈內

KO: 敲除

MSX: L-甲硫胺酸磺醯亞胺

MRTHL: 平均停留時間終端半衰期

NAM: 菸鹼醯胺

OEG: 8-胺基-3,6-二氧雜-辛酸

PBMC: 周邊血單核細胞

PCR: 聚合酶連鎖反應

pI: 等電點

PK: 藥物動力學

PO: 口服

ROUT: 穩健回歸和異常值去除（GraphPad Prism功能）

RMS: 均方根

RT-PCR: 反轉錄聚合酶鏈反應

RP-UPLC: 逆相超效液相層析

SIA: 序列一致類似物

SC: 皮下

SNAC： N-[8-(2-羥苯甲醯基)胺基]辛酸鈉

SPR: 表面電漿子共振

TGT: 凝血酶生成測定法

TLS: 平移-擺動-轉動-旋轉

TVT: 尾靜脈橫斷

UPLC: 超效液相層析

實例1：V_HH片段及其他重組蛋白之通用方法與製備

通用的分子生物學

【0286】 對於通用的分子生物學技術，參見Molecular Cloning: A Laboratory Manual (第4版，2014, Sambrook, Fritsch及Maniatis編，CSHL出版社，Cold Spring Harbor, NY USA)。

致免疫及文庫

【0287】 經Ablynx Camelid Facility倫理委員會批准(LA1400575)後，將兩隻美洲駝及兩隻羊駝分別以FIX及FX (Haemotologic Technologies, VT USA)致免疫。

【0288】 僅重鏈抗體片段所有組成成分的選殖及噬菌體免疫文庫的製備係如下進行。

【0289】 最後一次免疫原注射後，收集血液樣本。根據製造商的說明(Amersham Biosciences, Piscataway, NJ, US)，使用Ficoll-Hypaque從這些血液樣本中製備周邊血單核細胞(PBMC)。從PBMC中提取總RNA，並將其用作RT-PCR的起始材料，以擴增編碼V_HH片段的DNA區段，基本上如WO2005/044858所述。簡言之，將編碼V_HH片段的DNA區段選殖到噬菌粒載體pAX212中，該載體允許產生展示了與His_6-及$FLAG_3$-標籤融合的V_HH片段的噬菌體顆粒。隨後，根據標準實驗方案製備並儲存噬菌體。

合成文庫

【0290】 合成文庫係藉由而生成：將從致免疫所得到及選殖之合成V_HH基因片段選殖到噬菌粒載體pAX190中，該載體具有與上述pAX212相同的特徵，但在多個選殖位點上具有不同處。

與FX或FIX結合之V_HH片段的文庫篩選

【0291】 用生成的免疫及合成文庫進行了V_HH片段噬菌體展示選擇。針對不同濃度之固定化人類FIX及FX對文庫進行一至四輪連續增殖以富集殖株篩選，從而得到對於FIX與FX (Haemotologic Technologies, VT USA)及食蟹獼猴FIX與FX (Novo Nordisk公司自力生成)的較佳結合物。

【0292】 為了分別特異性地富集對FIXa與FX相對於FIX與FXa具有選擇性的V_HH片段，在某些實驗中，在培養具有固定化FIXa與FX之文庫期間使用過量的可溶性FIX與FXa進行競爭。

【0293】 為了特異性地富集對FIX與FX相對於其他結構上相關的凝血因子具有選擇性的V_HH片段，在某些實驗中，在培養具有固定化FIX與FX之文庫期間使用過量的可溶性FX與FIX進行競爭。

【0294】 針對ELISA（使用來自表現該V_HH片段的大腸桿菌細胞的周質萃取物）中對人類及食蟹獼猴FX、FXa、FIX及FIXa的結合，篩選了來自選擇輸出的大約4500個單獨的克隆。鑑認出大約1500個顯示與人類FX與FIXa/FIX特異性結合的殖株，其中大多數顯示與食蟹獼猴FX與FIX交叉結合。一些殖株顯示出與FXa相比優先結合FX且與FIX相比優先結合FIXa。對ELISA陽性殖株的序列分析鑑認出大約700個與FIXa/FIX或FX/FXa結合之V_HH片段的獨特序列。為了進一步優化合物，藉由通過使用凝血酶生成測定法進行突變篩選來著重止血效力的提高，通過篩選親和力改變的化合物來避免靶標介導的藥物傾向，通過使用MHC相關肽蛋白質體學測定篩選風險位點來降低免疫原性，以及通過降低表面暴露殘基的pI值來提高體內的口服生物可利用率，從而進行合併有延長子添加、連接子與延伸子合併之強化V_HH CDR及FR誘變。

與FX或FIX結合之V_HH片段的表現構築體生成

【0295】 來自噬菌體展示選擇輸出的V_HH片段的序列分析係按照眾所周知的程序來進行(Pardon等人，(2014) Nat Protoc 9: 674)。

【0296】 使用各自帶有獨特限制位點的正向FR1及反向FR4引物的特定組合，通過PCR獲得的含有V_HH片段的DNA片段，用適當的限制酶進行消化，並連接到V_HH多肽表現載體之匹配選殖匣中，作為大腸桿菌或畢氏酵母菌(*P. pastoris*)表現的His_6-及/或$FLAG_3$-標籤格式。接著將該連接混合物轉形至電勝任大腸桿菌TG1 (60502, Lucigen, Middleton, WI)或TOP10 (C404052, ThermoFisher Scientific, Waltham, MA)細胞中，接著使其在適當的抗生素選擇壓力(卡那黴素或Zeocin)下生長。通過質體DNA的Sanger定序(LGC Genomics，德國柏林)來驗證抗性殖株。

與FX或FIX結合之V_HH片段在大腸桿菌中的通用表現

【0297】 在大腸桿菌TG1中，從含有*lac*啟動子、卡那黴素抗性基因、大腸桿菌複製起點以及前面有OmpA信號肽編碼序列的V_HH片段選殖位點之質體表現載體表現V_HH片段。與V_HH多肽編碼序列符合讀框地，該載體編碼C末端$FLAG_3$和HIS_6標籤。信號肽將所表現的V_HH片段引導至細菌宿主的周質腔室。

【0298】 使所要之含有V_HH片段構築體的大腸桿菌TG-1細胞在含有「5052」自誘導培養基（0.5%甘油，0.05%葡萄糖，0.2%乳糖 + 3 mM $MgSO_4$）的帶擋板搖瓶中於37°C下生長2小時，之後在30°C下生長29小時。然後將來自大腸桿菌表現培養物的過夜冷凍細胞沉澱物溶解在PBS（原始培養物體積的1/12.5）中，並在輕輕旋轉下於4°C培養1小時。最後，將細胞再次沉澱，並儲存含有分泌到周質空間中的蛋白質的上清液。

V_HH片段在畢氏酵母菌(*P. pastoris*)中的通用表現

【0299】 使含有V_HH片段構築體的畢氏酵母細胞在BGCM培養基中生長

兩天(於30°C及200 rpm下)。在第三天，將培養基換成BMCM，並使構築體進一步生長(於30°C及200 rpm下)，並在8小時後用0.5% v/v甲醇進行誘導。第二天，在早晨、中午及晚上用0.5% v/v甲醇誘導構築體。在第五天，將細胞離心並收集上清液(含有分泌的V_HH片段)。

與FX或FIX結合之V_HH及抗體以及其他重組蛋白在HEK細胞中的通用表現

【0300】 用於在HEK293細胞中暫時表現的表現質體係購自Twist Biosciences公司或Thermo Fisher Scientific公司。購自Twist Biosciences公司的質體為Durocher, Y.等人，(2002) Nucleic Acid Res, 30: E9中所述之基於pTT載體的質體，而購自Thermo Fisher Scientific公司的質體為基於pcDNA34-Topo載體(Thermo Fisher Scientific)的質體。將具His_6-標籤或不具標籤的V_HH多肽化合物轉染至HEK293懸浮細胞中，以暫時表現V_HH多肽化合物。攜帶編碼抗GLA FIX及抗GLA FX抗體(Novo Nordisk公司自力生成)、艾美賽珠單抗(Hoffmann-La Roche股份有限公司，瑞士)序列相同類似物(SIA)及Mim8 (Novo Nordisk，丹麥)之序列的等校表現構築體係亦使用下述方法在HEK293中表現。

【0301】 HEK293懸浮細胞的暫時轉染（Expi293表現系統，Thermo Fisher Scientific，目錄編號A14635）基本上係按照製造商的說明進行。HEK293細胞通常在補充有1% P/S（GIBCO，目錄編號15140-122）之Expi293表現培養基（Gibco，目錄編號A14351-01）中每3-4天繼代一次。使用Expifectamine以每毫升2.5-3百萬個的細胞密度對HEK293細胞進行轉染。對於每公升HEK293細胞，轉染的方法是將總共1 mg的質體DNA稀釋到50 mL Optimem（GIBCO，目錄編號51985-026，稀釋物A），並將2.7 mL Expifectamine稀釋到50 mL Optimem（稀釋物B）。對於共轉染（即用於抗體），以1:1的比例使用質體。將稀釋物A及B混合並在室溫下培養10-20分鐘。此後將轉染混合物加入HEK293細胞中，並在37°C的加濕培養箱中

以軌道旋轉(85-140 rpm)的方式培養細胞。轉染一天後，將經轉染的細胞補充5 ml的ExpiFectamine 293轉染增強劑1及50 ml的ExpiFectamine 293轉染增強劑2。通常在轉染後4-5天通過離心後過濾來收取細胞培養上清液。

與FX及FIX(a)結合之V_HH片段及抗體在CHO細胞中的通用表現

【0302】 抗FIX及抗FX V_HH化合物及抗體係於中國倉鼠卵巢(CHO)細胞中使用麩醯胺合成酶(GS)選擇而產生。CHO細胞係以GS表現質體使用電穿孔進行轉染，此後在補充有MSX之CD-CHO培養基(Thermo Fisher Scientific，目錄編號10743029)中使用麩醯胺剝奪法進行選擇。通常在培養3週之後得到穩定CHO細胞池，之後將細胞池以單細胞選殖到384孔盤中。養在384孔盤中的CHO殖株通常被擴增到96孔盤並進行生產率篩選。

使用預定義的專用細胞培養基(Novo Nordisk A/S)，在生物反應器中以1公升至15公升的規模進行培養以將選定的生產殖株放大。在培養期間內，細胞存活率保持在高水平，隨後逐漸下降，直到收取細胞培養物。於此，在進行層析式蛋白質純化之前，以離心使細胞上清液澄清，及/或使用MD0HC23CL3與MX0HC01FS1過濾器(Millipore)進行深層式過濾，視培養規模而定。

與FX或FIX結合之V_HH片段之通用純化及定性

【0303】 具His6-標籤或不具標籤的V_HH化合物係藉由MabSelectSure蛋白質-A樹脂(Cytiva)或固定化金屬親和層析(IMAC)在Ni-Excel (Cytiva)樹脂上用咪唑(用於前者)或酸性溶析(用於後者)進行純化，接著進行脫鹽步驟(具Sephadex G25樹脂的PD管柱，Cytiva)，且在有必要時在PBS或HBS中進行凝膠過濾層析(Superdex200管柱，Cytiva)。不具His6-標籤的V_HH化合物及分別靶向FIX與FX之GLA結構域的抗體(Novo Nordisk公司自力生成)以及艾美賽珠單抗SIA

(Hoffmann-La Roche股份有限公司，瑞士)係藉由蛋白質-A樹脂MabSelectSure (Cytiva)或多峰樹脂(Cytiva)用酸性層析進行純化，接著進行脫鹽步驟(具Sephadex G25樹脂的PD管柱，Cytiva)，且在有必要時在PBS或HBS中進行凝膠過濾層析(Superdex200管柱，Cytiva)。蛋白質完整性係使用設置在Agilent LC 1100/1200系統上之高效粒徑排阻液相層析(SE-HPLC)方法並使用BIOSEP (用於分離生物分子的管柱)-SEC-53000 300×7.8 mm管柱(Phenomenex，目錄編號OOH-2146-K0)及由200 mM磷酸鈉(pH 6.9)、300 mM NaCl及10 %異丙醇所組成的運行緩衝液來進行分析。經純化的V_HH多肽批次之分子量係使用電噴霧電離飛行時間質譜法(ESI-TOF-MS)於6280 Agilent系統(Agilent Technologies)上用MassPREP脫鹽(Waters)管柱以每分鐘0.4 ml在由MQ-H2O/0.1%甲酸所組成的A緩衝液及由乙腈/0.1%甲酸所組成的B緩衝液進行階段式溶析來分析。使用NanoDrop™光譜儀(Thermo Scientific)及理論計算的消光係數來測量最終蛋白濃度。

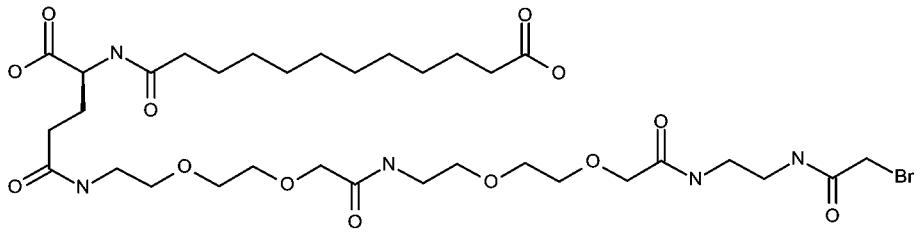
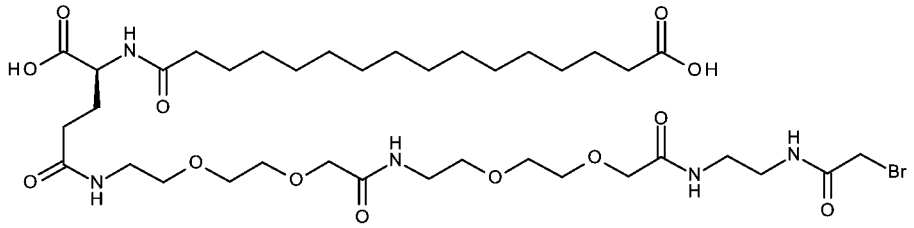
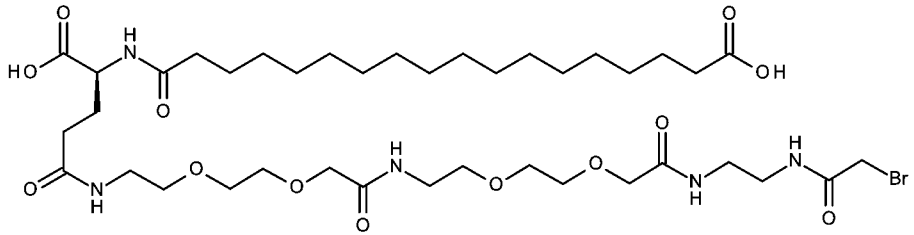
實例2：延長部分與V_HH多肽半胱胺酸殘基的共軛

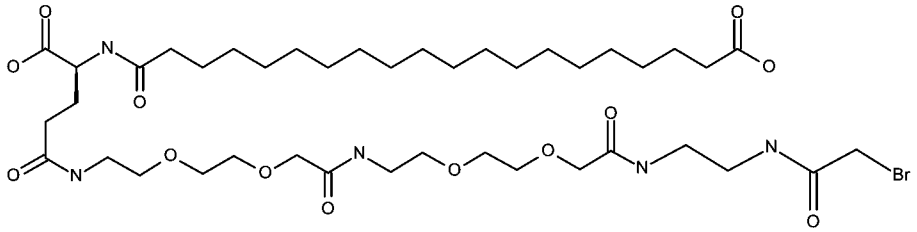
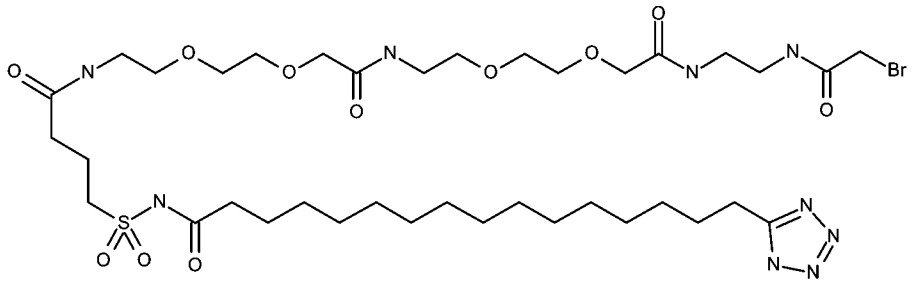
【0304】 為了延長V_HH多肽以改善藥物動力學特性，V_HH多肽化合物係經工程改造成在不同主鏈位置（例如在N端或C端延伸子）包含半胱胺酸(Cys)取代及一或兩個導入的Cys殘基，供與下文進一步描述的一或多個脂肪酸延長部分共軛。

【0305】 為了將延長部分接附至V_HH多肽化合物，使用呈修飾延長部分形式的中間反應物。

【0306】 該包含有延長部分之中間反應物係如WO2016/102562中所述製備，且這類中間反應物之非限制性實例係顯示於下表3中：

表3：包含有延長部分之中間反應物的實例（包括連接子(L_P)）

ID	*中間試劑結構及 IUPAC 名稱(簡稱)*	*V_HH 多肽衍生物 ID*
C1	 11-{(S)-1-羧基-3-[2-(2-{[2-(2-{[2-(2-溴乙醯基-胺基)乙基胺基甲醯基]甲氧基}乙氧基)乙基胺基甲醯基]甲氧基}乙氧基)乙基-胺基甲醯基]丙基胺基甲醯基}十一酸（C12 二酸連接子試劑）	化合物#65
C2	 15-{(S)-1-羧基-3-[2-(2-{[2-(2-{[2-(2-溴乙醯基-胺基)乙基胺基甲醯基]甲氧基}乙氧基)乙基胺基甲醯基]甲氧基}乙氧基)乙基-胺基甲醯基]丙基胺基甲醯基}十五酸（C16 二酸連接子試劑）	化合物#6、#7、#8、#9、#10、#11、#12、#13、#14、#15、#16、#17、#18、#19、#20、#21、#22
C3	 17-{(S)-1-羧基-3-[2-(2-{[2-(2-{[2-(2-溴乙醯基-胺基)乙基胺基甲醯基]甲氧基}乙氧基)乙基胺基甲醯基]甲氧基}乙氧基)乙基-胺基甲醯基]丙基胺基甲醯基}十七酸（C18 二酸連	化合物#4、#5、#24、#26、#28、#30、#32、#34、#36、#38、#40、#58、#59、#60、#61、#62、#63、#66

	接子試劑）	
C4	 19-{(S)-1-羧基-3-[2-(2-{[2-(2-{[2-(2-溴乙醯基-胺基)乙基胺基甲醯基]甲氧基}乙氧基)乙基胺基甲醯基]甲氧基}乙氧基)乙基-胺基甲醯基]丙基胺基甲醯基}十九酸（C20 二酸連接子試劑）	化合物#67
C5	 N-[4-[2-[2-[2-[2-[2-[2-[2-[(2-溴乙醯基)胺基]乙基胺基]-2-側氧基-乙氧基]乙氧基]乙基胺基]-2-側氧基-乙氧基]乙氧基]乙基胺基]-4-側氧基-丁基]磺醯基-16-(1H-四唑-5-基)十六醯胺（四唑連接子試劑）	化合物#68

共軛、純化及分析

【0307】 向含有帶著共軛用之一或多個導入半胱胺酸殘基之V_HH多肽的水溶液添加5當量的BSPP (雙(對-磺酸根基苯基)苯基膦二水合物二鉀鹽)或1.1當量之以半胱胺酸封端的TCEP (三(2-羧基乙基)鹽酸膦)。攪拌1至2小時後，用NaOH調整pH至8.5，且個別V_HH多肽中每個游離半胱胺酸添加5當量之於0.1 M $NaHCO_3$水溶液中的中間反應物C1。在暗室中溫和攪拌該混合物1.5至16小時。將反應混合物用水稀釋，之後使用Äkta系統以陰離子交換(AIEX)法進行純化。帶側鏈共軛的V_HH多肽係使用AIEX層析分離法進行純化。因此，裝填於適宜管柱中之AIEX樹脂Source 30Q係與安裝在Äkta Avant層析系統上之氯化鈉梯度程式一起使用。所使用之緩衝液系統為由20 mM Tris (pH 8.5)所組成之平衡緩衝液以及由20 mM Tris、1 M NaCl (pH 8.5)所組成之溶析緩衝液。將該反應混合物調整至pH 8.5，且使用MilliQ水或平衡緩衝液將傳導度降低到至4 mS/cm。將樣本施用於管柱上且在施用後用5至10倍管柱體積的平衡緩衝液沖洗管柱。接著使用淺變梯度之30至50倍管柱體積進行分離層析。所使用的梯度係從0%至50%，視所純化之V_HH多肽衍生物的pI而定。一般而言，未共軛的親本V_HH多肽會在梯度早期先溶離出，單共軛的V_HH多肽會在梯度中期溶離出，具多重共軛的V_HH多肽（指每個V_HH多肽分子有超過一個側鏈共軛）則在梯度後期溶離出。將主峰餾分進行匯集，以得到90%至99%之單共軛或雙共軛的高純度V_HH多肽製劑。使用逆相超效液相層析(RP-UPLC)，以設置在具有UV及FLD感測器之Waters Acquity UPLC系統上的HALO二苯基管柱1000 Å, 2.7 µm, 150x2.1 mm (Scantec Nordic USDPF001316)以及由A) 0.1% v/v TFA水溶液與B) 0.09% v/v TFA乙腈溶液所組成之運行緩衝液進行純度分析。管柱溫度設定為60°C。梯度程式為：1) 0.0-8.0 min: 20-50% B，2) 8.0-8.1 min: 50-80% B，3) 8.1-9.0 min: 80% B，4) 9.0-9.1 min: 80-20% B，及5) 9.1-11.0 min: 20% B。未共軛的親本V_HH多肽會在4.6至4.8分鐘溶

離出。單共軛的V_HH多肽衍生物會在5.1至5.6分鐘溶離出，且主峰在大約5.3分鐘處。具雙共軛的V_HH多肽衍生物會在5.7至5.8分鐘溶離出，後來是具位點外多重共軛的V_HH多肽。帶共軛的V_HH多肽完整性係使用建立在Agilent LC 1100/1200系統及BIOSEP-SEC-3000 300×7.8 mm管柱(Phenomenex，目錄編號OOH-2146-K0)及由200 mM磷酸鈉(pH 6.9)、300 mM NaCl及10 %異丙醇所組成的運行緩衝液上之SE-HPLC方法進行分析。帶共軛的V_HH多肽之分子量係使用ESI-TOF-MS於6280 Agilent系統(Agilent Technologies)上用MassPREP脫鹽(Waters)管柱以每分鐘0.4 ml在由MilliQ水/0.1%甲酸所組成的A緩衝液及由乙腈/0.1%甲酸所組成的B緩衝液進行階段式溶析來分析。使用基於胰凝乳蛋白酶與胰蛋白酶的消化酶組合進行肽定位以驗證序列。LC-MS系統包括一個Waters Aquity UPLC和一個Thermo Orbitrap Fusion儀器。消化酶的線上LC-MS分析係使用CSH C-18管柱1.7 µm, 150x2.1 mm和乙腈/甲酸梯度進行。管柱子溫度為60C。緩衝液A：0,1%FA水，緩衝液B：0,1%FA乙腈。梯度程式為：1）0.0-2.0分鐘：1% B，2）2-50分鐘：1-35% B，3）50-51分鐘：100%B，以及4）51-60分鐘：100-1% B，流量為120µl/分鐘。使用Genedata Refiner肽定位工作流程對資料進行分析。獲得主序列的全部範圍。使用NanoDrop™光譜儀(Thermo Scientific)以理論計算的消光係數來測量分批製備之帶共軛的V_HH多肽衍生物的蛋白濃度。

【0308】 圖1、2及3係顯示越來越多細節的ISVD及V_HH多肽衍生物之非限制性實例。

實例3：序列及抗FX/抗FIX(A) V_HH多肽列表

表4：化合物及不具延長子之SEQ ID NO (V_HH & CDR)概覽

	N 端延伸子	抗 FX V_HH (N 端)		連接子 (L_{1-2})	抗 FIX(a) V_HH (C 端)		C 端延伸子
V_HH 多肽 ID	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**
化合物#1 (單價)	無	VHH-1.1	27 (28,29,30)	無	無	無	5
化合物#2 (單價)	無	無	無	無	VHH-2.2	35 (36,37,38)	6
化合物#3 (單價)	無	VHH-1.3	31 (32,33,34)	無	無	無	6
化合物#4	無	VHH-1.4	39 (40,41,42)	14	VHH-2.4	43 (44,45,46)	7
化合物#5	無	VHH-1.5	47 (48,49,50)	14	VHH-2.5	51 (52,53,54)	7
化合物#6	無	VHH-1.6	47 (48,49,50)	14	VHH-2.6	51 (52,53,54)	8
化合物#7	無	VHH-1.7	63 (64,65,66)	20	VHH-2.7	67 (68,69,70)	9
化合物#8	無	VHH-1.8	63 (64,65,66)	20	VHH-2.8	51 (52,53,54)	9
化合物#9	無	VHH-1.9	79 (80,81,82)	20	VHH-2.9	83 (84,85,86)	9
化合物#10	無	VHH-1.10	87 (88,89,90)	20	VHH-2.10	67 (68,69,70)	9
化合物#11	無	VHH-1.11	95 (96,97,98)	20	VHH-2.11	83 (84,85,86)	9
化合物#12	無	VHH-1.12	103 (104,105,106)	20	VHH-2.12	107 (108,109,110)	9

	N 端延伸子	抗 FX V_HH (N 端)		連接子 (L_{1-2})	抗 FIX(a) V_HH (C 端)		C 端延伸子
V_HH 多肽 ID	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**
化合物#13	無	VHH-1.13	111 (112,113,114)	20	VHH-2.13	115 (116,117,118)	9
化合物#14	無	VHH-1.14	119 (120,121,122)	20	VHH-2.14	123 (124,125,126)	9
化合物#15	無	VHH-1.15	127 (128,129,130)	20	VHH-2.15	131 (132,133,134)	9
化合物#16	無	VHH-1.16	111 (112,113,114)	20	VHH-2.16	139 (140,141,142)	9
化合物#17	無	VHH-1.17	143 (144,145,146)	20	VHH-2.17	107 (108,109,110)	9
化合物#18	無	VHH-1.18	151 (152,153,154)	20	VHH-2.18	155 (156,157,158)	9
化合物#19	無	VHH-1.19	159 (160,161,162)	20	VHH-2.19	51 (52,53,54)	9
化合物#20	無	VHH-1.20	167 (168,169,170)	20	VHH-2.20	171 (172,173,174)	9
化合物#21	無	VHH-1.21	79 (80,81,82)	20	VHH-2.21	107 (108,109,110)	9
化合物#22	無	VHH-1.22	111 (112,113,114)	20	VHH-2.22	83 (84,85,86)	9
化合物#23	690	VHH-1.23	191 (192,193,194)	14	VHH-2.23	43 (44,45,46)	10
化合物#24	690	VHH-1.24	191 (192,193,194)	14	VHH-2.24	43 (44,45,46)	10
化合物#25	690	VHH-1.25	207 (208,209,210)	14	VHH-2.25	43 (44,45,46)	11

	N 端延伸子	抗 FX V_HH (N 端)		連接子 (L_{1-2})	抗 FIX(a) V_HH (C 端)		C 端延伸子
V_HH 多肽 ID	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**
化合物#26	690	VHH-1.26	207 (208,209,210)	14	VHH-2.26	43 (44,45,46)	11
化合物#27	690	VHH-1.27	223 (224,225,226)	14	VHH-2.27	227 (228,229,230)	11
化合物#28	690	VHH-1.28	223 (224,225,226)	14	VHH-2.28	227 (228,229,230)	11
化合物#29	690	VHH-1.29	223 (224,225,226)	14	VHH-2.29	243 (244,245,246)	11
化合物#30	690	VHH-1.30	223 (224,225,226)	14	VHH-2.30	243 (244,245,246)	11
化合物#31	690	VHH-1.31	223 (224,225,226)	14	VHH-2.31	259 (260,261,262)	11
化合物#32	690	VHH-1.32	223 (224,225,226)	14	VHH-2.32	259 (260,261,262)	11
化合物#33	690	VHH-1.33	223 (224,225,226)	14	VHH-2.33	275 (276,277,278)	11
化合物#34	690	VHH-1.34	223 (224,225,226)	14	VHH-2.34	275 (276,277,278)	11
化合物#35	690	VHH-1.35	223 (224,225,226)	14	VHH-2.35	291 (292,293,294)	11
化合物#36	690	VHH-1.36	223 (224,225,226)	14	VHH-2.36	291 (292,293,294)	11
化合物#37	690	VHH-1.37	223 (224,225,226)	14	VHH-2.37	307 (308,309,310)	11
化合物#38	690	VHH-1.38	223 (224,225,226)	14	VHH-2.38	307 (308,309,310)	11

	N 端延伸子	抗 FX V_HH (N 端)		連接子 (L_{1-2})	抗 FIX(a) V_HH (C 端)		C 端延伸子
V_HH 多肽 ID	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**
化合物#39	690	VHH-1.39	223 (224,225,226)	14	VHH-2.39	323 (324,325,326)	11
化合物#40	690	VHH-1.40	223 (224,225,226)	14	VHH-2.40	323 (324,325,326)	11
化合物#41	無	VHH-1.41	335 (336,337,338)	16	VHH-2.41	51 (52,53,54)	7
化合物#42	無	VHH-1.42	335 (336,337,338)	17	VHH-2.42	51 (52,53,54)	7
化合物#43	無	VHH-1.43	335 (336,337,338)	18	VHH-2.43	51 (52,53,54)	7
化合物#44	無	VHH-1.44	335 (336,337,338)	19	VHH-2.44	51 (52,53,54)	7
化合物#45	無	VHH-1.45	335 (336,337,338)	20	VHH-2.45	51 (52,53,54)	7
化合物#46	無	VHH-1.46	335 (336,337,338)	14	VHH-2.46	379 (380,381,382)	7
化合物#47	無	VHH-1.47	335 (336,337,338)	16	VHH-2.47	379 (380,381,382)	7
化合物#48	無	VHH-1.48	335 (336,337,338)	21	VHH-2.48	379 (380,381,382)	7
化合物#49	無	VHH-1.49	335 (336,337,338)	18	VHH-2.49	379 (380,381,382)	7
化合物#50	無	VHH-1.50	335 (336,337,338)	19	VHH-2.50	379 (380,381,382)	7
化合物#51	無	VHH-1.51	335 (336,337,338)	14	VHH-2.51	419 (420,421,422)	7

	N 端延伸子	抗 FX V_HH (N 端)		連接子 (L_{1-2})	抗 FIX(a) V_HH (C 端)		C 端延伸子
V_HH 多肽 ID	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**
化合物#52	無	VHH-1.52	335 (336,337,338)	16	VHH-2.52	419 (420,421,422)	7
化合物#53	無	VHH-1.53	335 (336,337,338)	23	VHH-2.53	419 (420,421,422)	7
化合物#54	無	VHH-1.54	335 (336,337,338)	21	VHH-2.54	419 (420,421,422)	7
化合物#55	無	VHH-1.55	335 (336,337,338)	18	VHH-2.55	419 (420,421,422)	7
化合物#56	無	VHH-1.56	335 (336,337,338)	19	VHH-2.56	419 (420,421,422)	7
化合物#57	無	VHH-1.57	335 (336,337,338)	20	VHH-2.57	419 (420,421,422)	7
化合物#58	無	VHH-1.58	471 (472,473,474)	14	VHH-2.58	475 (476,477,478)	7
化合物#59	無	VHH-1.59	479 (480,481,482)	14	VHH-2.59	51 (52,53,54)	7
化合物#60	無	VHH-1.60	487 (488,489,490)	14	VHH-2.60	475 (476,477,478)	7
化合物#61	無	VHH-1.61	495 (496,497,498)	14	VHH-2.61	499 (500,501,502)	7
化合物#62	無	VHH-1.62	503 (504,505,506)	14	VHH-2.62	507 (508,509,510)	7
化合物#63	無	VHH-1.63	511 (512,513,514)	14	VHH-2.63	507 (508,509,510)	7
化合物#64	690	VHH-1.64	223 (224,225,226)	14	VHH-2.64	43 (44,45,46)	10

	N 端延伸子	抗 FX V_HH (N 端)		連接子 (L_{1-2})	抗 FIX(a) V_HH (C 端)		C 端延伸子
V_HH 多肽 ID	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**
化合物#65	690	VHH-1.65	223 (224,225,226)	14	VHH-2.65	43 (44,45,46)	10
化合物#66	690	VHH-1.66	223 (224,225,226)	14	VHH-2.66	43 (44,45,46)	10
化合物#67	690	VHH-1.67	223 (224,225,226)	14	VHH-2.67	43 (44,45,46)	10
化合物#68	690	VHH-1.68	223 (224,225,226)	14	VHH-2.68	43 (44,45,46)	10
化合物#69	無	VHH-1.69	39 (40,41,42)	14	VHH-2.69	43 (44,45,46)	7
化合物#70	3	VHH-1.70	223 (224,225,226)	14	VHH-2.70	43 (44,45,46)	11
化合物#71	4	VHH-1.71	223 (224,225,226)	14	VHH-2.71	43 (44,45,46)	11
化合物#72	無	VHH-1.72	223 (224,225,226)	14	VHH-2.72	43 (44,45,46)	12
化合物#73	無	VHH-1.73	223 (224,225,226)	14	VHH-2.73	43 (44,45,46)	13
化合物#74	690	VHH-1.74	599 (600,601,602)	691	VHH-2.74	603 (604,605,606)	7
化合物#75	無	VHH-1.75	607 (608,609,610)	14	VHH-2.75	35 (36,37,38)	無
化合物#76 (單價)	無	VHH-1.15	127 (128,129,130)	無	無	無	11
化合物#77	無	VHH-1.13	111 (112,113,114)	無	無	無	11

	N 端延伸子	抗 FX V_HH (N 端)		連接子 (L_{1-2})	抗 FIX(a) V_HH (C 端)		C 端延伸子
V_HH 多肽 ID	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**	**V_HH ID**	**SEQ ID NO** V_HH (CDR1, 2, 3) *(Kabat 定義)*	**SEQ ID NO**
(單價)							

表5：抗FX/抗FIX(a) V_HH多肽衍生物（包括延長部分）概覽

V_HH 多肽 ID	延長子 **(P)**	延長部分連接子 **(L_P)**	延長部分接附位點(“R1(/R2)”)	化合物#主鏈 **SEQ ID NO** (不含延長部分)
化合物#1	無	無	無	615
化合物#2	無	無	無	616
化合物#3	無	無	無	617
化合物#4	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	618
化合物#5	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	619
化合物#6	P2	L_P1	C 端延伸的 Cys5 及 Cys7 (SEQ ID NO:8)	620
化合物#7	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	621
化合物#8	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	622
化合物#9	P2	L_P1	C 端延伸的 Cys4 及 Cys6	623

V_HH 多肽 ID	延長子 (P)	延長部分連接子 (L_P)	延長部分接附位點(“R1(/R2)”)	化合物#主鏈 SEQ ID NO (不含延長部分)
			(SEQ ID NO:9)	
化合物#10	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	624
化合物#11	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	625
化合物#12	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	626
化合物#13	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	627
化合物#14	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	628
化合物#15	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	629
化合物#16	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	630
化合物#17	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	631
化合物#18	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	632
化合物#19	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	633
化合物#20	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	634
化合物#21	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	635
化合物#22	P2	L_P1	C 端延伸的 Cys4 及 Cys6 (SEQ ID NO:9)	636

V_HH 多肽 ID	延長子 (P)	延長部分連接子 (L_P)	延長部分接附位點(“R1(/R2)”)	化合物#主鏈 SEQ ID NO (不含延長部分)
化合物#23	無	無	無	637
化合物#24	P3	L_P1	C 端延伸的 Cys10 (SEQ ID NO:10)	638
化合物#25	無	無	無	639
化合物#26	P3	L_P1	化合物#26 中的 Cys30 (SEQ ID NO:640)	640
化合物#27	無	無	無	641
化合物#28	P3	L_P1	化合物#28 中的 Cys158 (SEQ ID NO:642)	642
化合物#29	無	無	無	643
化合物#30	P3	L_P1	化合物#30 中的 Cys160 (SEQ ID NO:644)	644
化合物#31	無	無	無	645
化合物#32	P3	L_P1	化合物#32 中的 Cys161 (SEQ ID NO:646)	646
化合物#33	無	無	無	647
化合物#34	P3	L_P1	化合物#34 中的 Cys188 (SEQ ID NO:648)	648
化合物#35	無	無	無	649
化合物#36	P3	L_P1	化合物#36 中的 Cys190 (SEQ ID NO:650)	650
化合物#37	無	無	無	651
化合物#38	P3	L_P1	化合物#38 中的 Cys209 (SEQ ID NO:652)	652

V_HH 多肽 ID	延長子 (P)	延長部分連接子 (L_P)	延長部分接附位點(“R1(/R2)”)	化合物#主鏈 SEQ ID NO (不含延長部分)
化合物#39	無	無	無	653
化合物#40	P3	L_P1	化合物#40 中的 Cys231 (SEQ ID NO:654)	654
化合物#41	無	無	無	655
化合物#42	無	無	無	656
化合物#43	無	無	無	657
化合物#44	無	無	無	658
化合物#45	無	無	無	659
化合物#46	無	無	無	660
化合物#47	無	無	無	661
化合物#48	無	無	無	662
化合物#49	無	無	無	663
化合物#50	無	無	無	664
化合物#51	無	無	無	665
化合物#52	無	無	無	666
化合物#53	無	無	無	667
化合物#54	無	無	無	668
化合物#55	無	無	無	669
化合物#56	無	無	無	670

V_HH 多肽 ID	**延長子 (P)**	**延長部分連接子 (L_P)**	**延長部分接附位點(“R1(/R2)”)**	**化合物#主鏈 SEQ ID NO (不含延長部分)**
化合物#57	無	無	無	671
化合物#58	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	672
化合物#59	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	673
化合物#60	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	674
化合物#61	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	675
化合物#62	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	676
化合物#63	P3	L_P1	C 端延伸的 Cys6 (SEQ ID NO:7)	677
化合物#64	無	無	無	678
化合物#65	P1	L_P1	C 端延伸的 Cys10 (SEQ ID NO:10)	679
化合物#66	P3	L_P1	C 端延伸的 Cys10 (SEQ ID NO:10)	680
化合物#67	P4	L_P1	C 端延伸的 Cys10 (SEQ ID NO:10)	681
化合物#68	P5	L_P2	C 端延伸的 Cys10 (SEQ ID NO:10)	682
化合物#69	無	無	無	683
化合物#70	P6 (“P”-“R1”)	L_P3 (“P”-“L_P”-“R1”)	VHH-1.70 中的 Asp1 (SEQ ID NO:567)	684
化合物#71	P6 (“P”-“R1”)	L_P4 (“P”-“L_P”-“R1”)	VHH-1.71 中的 Asp1	685

V_HH 多肽 ID	延長子 **(P)**	延長部分連接子 **(L_P)**	延長部分接附位點(“R1(/R2)”)	化合物#主鏈 **SEQ ID NO** (不含延長部分)
			(SEQ ID NO:575)	
化合物#72	P6 (“R1”-“P”)	L_P3 (“R1”-“L_P”-“P”)	VHH-2.72 中的 Ser127 (SEQ ID NO:587)	686
化合物#73	P6 (“R1”-“P”)	L_P4 (“R1”-“L_P”-“P”)	VHH-2.73 中的 Ser127 (SEQ ID NO:595)	687
化合物#74	無	無	無	688
化合物#75	無	無	無	689

實例4：基於ELISA實驗的抗FX V_HH多肽表位

【0309】 為了判定命名為「化合物#1」之抗FX V_HH多肽的結合表位是否位於人類FX (SEQ ID NO:2)的活化肽(AP)內，建立了ELISA測定。將41個以一個胺基酸間距跨越該活化肽的52個殘基之獨特12-聚體肽片段固定在微量滴定盤孔中，隨後與待測的V_HH多肽一起培養，並通過加入HRP標記的第二抗體對結合的配體進行檢測。

【0310】 肽在C末端與生物素共軛，並使用50 μL的1 μg/mL肽溶液固定在預先用1 μg/mL鏈黴親和素塗覆的微量滴定盤的不連續孔中。每個孔用洗滌緩衝液(10mM Tris, 150mM NaCl, 2.5mM $CaCl_2$, 0.05% Tween 20, pH 8.60)洗滌，隨後添加50 μL的2 μg/mL FLAG標籤的待測抗FX V_HH多肽或IgG抗體。1小時後使用洗滌緩衝液洗去未結合的V_HH多肽。

【0311】 如下檢測結合的抗FX活化肽配體：首先結合HRP標記的第二抗體（對於FLAG標籤的V_HH多肽：抗FLAG mAb M2-過氧化物酶(HRP)(Sigma Aldrich, US) 1小時，然後加入100 μL TMB-1 ELISA受質(Kem-En-Tec Diagnostics，丹麥）。

【0312】 然後通過鑑定被每種FX結合物結合的肽組(表6)所覆蓋的共同序列，從產生高於基線的信號的這組肽中推導出最小表位。表位的第一個殘基由第一個連續的ELISA陽性肽中的最後一個胺基酸來定義，而表位的最後一個殘基被定義為最後一個連續的ELISA陽性肽的第一個殘基。

【0313】 因此，下表顯示了對於相應於化合物#1之初始檢測的抗FX V_HH多肽，來自跨越整個FX AP的連續肽的ELISA信號。ELISA信號已相對於背景信號進行了歸一化。

【0314】 灰色區域標記出用於判定最小表位的陽性結合信號，其被鑑定為序列NPFDLLDF (SEQ ID NO:692)。

【0315】 將化合物#1被鑑定出的FX活化肽之表位序列用於表位:互補位交互作用之高解析結構判定實驗，如實例6中概述。

表6：表位鑑定

表位殘基(SEQ ID NO)	**化合物#1**
空	1.00
SVAQATSSSGEA (693)	0.96
VAQATSSSGEAP (694)	1.02
AQATSSSGEAPD (695)	0.97
QATSSSGEAPDS (696)	0.96
ATSSSGEAPDSI (697)	0.98
TSSSGEAPDSIT (698)	0.77
SSSGEAPDSITW (699)	0.86
SSGEAPDSITWK (700)	0.88
SGEAPDSITWKP (701)	0.88
GEAPDSITWKPY (702)	0.93
EAPDSITWKPYD (703)	0.97
APDSITWKPYDA (704)	1.06
PDSITWKPYDAA (705)	1.05
DSITWKPYDAAD (706)	1.08
SITWKPYDAADL (707)	1.08
ITWKPYDAADLD (708)	1.10
TWKPYDAADLDP (709)	1.13
WKPYDAADLDPT (710)	0.89
KPYDAADLDPTE (711)	0.80
PYDAADLDPTEN (712)	0.83
YDAADLDPTENP (713)	0.87
DAADLDPTENPF (714)	0.88
AADLDPTENPFD (715)	0.88
ADLDPTENPFDL (716)	0.91
DLDPTENPFDLL (717)	1.00
LDPTENPFDLLD (718)	1.11
DPTENPFDLLDF (719)	4.16
PTENPFDLLDFN (720)	4.58
TENPFDLLDFNQ (721)	4.84
ENPFDLLDFNQT (722)	4.00
NPFDLLDFNQTQ (723)	3.13
PFDLLDFNQTQP (724)	4.95
FDLLDFNQTQPE (725)	4.38
DLLDFNQTQPER (726)	3.08
LLDFNQTQPERG (727)	1.88
LDFNQTQPERGD (728)	0.97
DFNQTQPERGDN (729)	0.94
FNQTQPERGDNN (730)	1.00
NQTQPERGDNNL (731)	0.92
QTQPERGDNNLT (732)	0.88
TQPERGDNNLTR (733)	1.05

實例5：抗FIX V_HH片段化合物#2之結晶及互補位/表位定位

【0316】 此研究之目的為判定命名為化合物#2之V_HH片段的互補位及表位殘基。

結晶化

【0317】 與人類EGR-CMK-經抑制因子IXa無Gla域(野生型)(購自Cambridge ProteinWorks批號hGDFIXAWTEGR_11)以1:1莫耳比率混合的V_HH多肽化合物#2之結晶係使用坐滴蒸氣擴散技術於18°C下生長。將100 nl的8.4 mg/ml複合物於20 mM Tris-HCl (pH 7.4)、50mM NaCl、及2.5 mM $CaCl_2$中的蛋白質溶液與100 nl的0.2 M硫酸鋰、0.1 M Tris-HCl (pH 8.5)、30% (w/v) PEG 4000 (作為沈澱劑)混合並以60 μl沈澱劑培養。在兩星期內出現結晶。

繞射數據收集

【0318】 結晶係於在液態氮中急速冷卻之前藉由將1 μl加有20%的乙二醇的沈澱劑加至結晶滴而冷凍保護。繞射數據係以100K於Swiss Light Source光束線X06DA (1.0000 Å波長)使用來自Dectris的Pilatus 2M像素偵測器收集。數據之自動索引、集成及縮放係使用來自XDS套裝的程式執行(繞射數據統計係於表7中概述)。

結構判定及精修

【0319】 如由Matthews係數分析所判斷，不對稱單元含有兩個化合物#2:EGR-CMK-經抑制因子IXa無Gla域複合物。結構係藉由分子置換測定，使用如於程式套裝Phenix中實施的Phaser，並使用一預定的V_HH:FIXa複合物結構做為搜尋模型。該正確胺基酸序列係使用COOT建立模型且之後使用Phenix精修步驟及COOT中的手動重建進行精修。精修統計可在表7中找到。

表7：數據收集及精修統計

波長(Å)	1.0000
解析度範圍(Å)	43.26-2.7 (2.797 - 2.7)
空間群	P 1
晶格(Å, deg)	56.51 64.47 64.72 76.115 87.449 85.761
總反射數	42366 (4277)
獨立反射數	23473 (2340)
多重度	1.8 (1.8)
完整度(%)	96.66 (96.77)
平均訊雜比強度 I/σ(I)	6.44 (1.40)
Wilson B-因子($Å^2$)	40.75
合併的 R 因子	0.1042 (0.5247)
非重複性合併的 R 因子	0.1473 (0.7416)
精確指標合併的 R 因子	0.1041 (0.5241)
CC1/2	0.986 (0.594)
CC*	0.997 (0.863)
用於精修的反射數	23455 (2340)
用於計算自由 R 因子的反射數	1176 (115)
工作 R 因子	0.2260 (0.2951)
自由 R 因子	0.2825 (0.3665)
CC(工作)	0.922 (0.745)
CC(自由)	0.829 (0.421)
非氫原子之原子數目	6622
巨分子	6394
配體	54
溶劑	174
蛋白質殘基	823
RMS(鍵長) (Å)	0.004
RMS(鍵角) (deg)	0.87
拉氏圖(Ramachandran)偏好比例(%)	94.82
拉氏圖允許比例(%)	5.18
拉氏圖極端比例(%)	0.00
離群旋轉異構體(%)	6.39
對撞分數	9.91
平均 B-因子($Å^2$)	42.93
巨分子	43.06
配體	42.44
TLS 群數目	38.49

對於最高解析殼之統計係顯示於括弧中。

表位及互補位之判定

【0320】 化合物#2的表位（其定義為特徵為在離V_HH多肽中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的FIX(a)殘基）係包含以下來自FIX(a)蛋白酶結構域的殘基：

【0321】 根據FIX序列（連續編號）(SEQ ID NO:1)之E224、T225、G226、V250、I251、R252、I253、P255、H257及N260。

【0322】 化合物#2的互補位（其定義為特徵為在離FIX(a)中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的殘基）係包含以下來自化合物#2的殘基：

F29、N30、Y32、T54、D99、R100、S101、F102、L103、F104、Q106、A107及N113 (SEQ ID NO:35)（連續編號）。

實例6：抗FX V_HH片段化合物#1之結晶及互補位/表位定位

【0323】 此研究之目的為判定命名為化合物#1之V_HH片段的互補位及表位殘基。

結晶化

【0324】 與實例4中所鑑定之對應於人類FX活化肽序列aa31-37之合成肽N-端-NPFDLLD-C-端(購自Schafer-N ApS)以1:14莫耳比率混合的V_HH多肽化合物#1之結晶係使用坐滴蒸氣擴散技術於18°C下生長。將150 nl的4.0 mg/ml複合物於20 mM Tris-HCl (pH 7.4)、50mM NaCl中的蛋白質溶液與50 nl的0.2 M NaCl、2 M硫酸銨、0.1 M二甲胂酸鈉(pH 6.5) (作為沈澱劑)混合並以60 µl沈澱劑培養。在一星期內出現結晶。

繞射數據收集

【0325】 結晶係於在液態氮中急速冷卻之前藉由將1 µl加有20%的乙二醇的沈澱劑加至結晶滴而冷凍保護。繞射數據係以100K於Swiss Light Source光束

線X06DA (1.0000 Å波長)使用來自Dectris的Pilatus 2M像素偵測器收集。數據之自動索引、集成及縮放係使用來自XDS套裝的程式執行(繞射數據統計係於表8中概述)。

結構判定及精修

【0326】 不對稱單元含有一個化合物#1:肽複合物。結構係藉由分子置換測定，使用如於程式套裝Phenix中實施的Phaser，並使用名為Protein Data Bank ID 4B41 (鏈A)之V_HH多肽結構做為搜尋模型。導入化合物#1的正確胺基酸序列，鑑定出該合成肽的差異電子密度並手動建立模型，且全使用COOT。此後使用Phenix精修及COOT手動重建的步驟來精修結構。精修統計可在表8中找到。

表8：數據收集及精修統計

波長(Å)	1.0000
解析度範圍(Å)	47.61-3.183 (3.297-3.183)
空間群	$P6_122$
晶格(Å, deg)	83.65 83.65 126.32 90 90 120
總反射數	80989 (3197)
獨立反射數	4667 (353)
多重度	17.4 (9.1)
完整度(%)	97.57 (76.08)
平均訊雜比強度 I/σ(I)	8.69 (1.55)
Wilson B-因子(Å^2)	50.39
合併的 R 因子	0.4146 (1.447)
非重複性合併的 R 因子	0.4271 (1.516)
精確指標合併的 R 因子	0.1004 (0.4227)
CC1/2	0.985 (0.651)
CC*	0.996 (0.888)
用於精修的反射數	4664 (353)
用於計算自由 R 因子的反射數	234 (18)
工作 R 因子	0.1948 (0.2689)
自由 R 因子	0.2500 (0.3981)
CC(工作)	0.957 (0.685)
CC(自由)	0.934 (0.389)
非氫原子之原子數目	986
巨分子	981

配體	5
溶劑	0
蛋白質殘基	130
RMS(鍵長) (Å)	0.003
RMS(鍵角) (deg)	0.59
拉氏圖(Ramachandran)偏好比例(%)	96.83
拉氏圖允許比例(%)	3.17
拉氏圖極端比例(%)	0.00
離群旋轉異構體(%)	0.00
對撞分數	8.68
平均 B-因子(Å^2)	44.41
巨分子	44.18
配體	88.79
TLS 群數目	1

對於最高解析殼之統計係顯示於括弧中。

表位及互補位之判定

【0327】 根據對應之連續編號的FX活化肽序列(SEQ ID NO:2)，該合成肽的表位（其定義為特徵為在離V_HH多肽化合物#1中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的FX活化肽殘基）係包含以下來自合成肽N173、P174、F175、L177及L178的殘基。

【0328】 V_HH多肽化合物#1的互補位（其定義為特徵為在離合成肽中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的化合物#1殘基）係包含以下來自化合物#1的殘基：

D32、A33、M34、G35、Y37、L47、V48、A49、G50、I51、M52、N57、T58、N59、Y60、T61、K97、V99、R101及P102 (SEQ ID NO:27)（連續編號）。

【0329】 由於化合物#3被用作親本V_HH多肽序列以基於序列及結構的合理優化，進行了與合成肽複合的V_HH多肽化合物#3的同源模型的構建。

【0330】 上述實驗中化合物#1與合成肽複合的結晶結構係用作化合物#3

與合成肽複合的同源模型之起始模型。比對化合物#1及化合物#3的胺基酸序列，並在COOT中對這兩個序列之間不同的胺基酸殘基進行突變，以建立化合物#3的起始模型。此模型（包括化合物#3）係經過預處理、優化，並在SCHRÖDINGER公司的MAESTRO中通過有限最小化運行。

【0331】 由於這些位置的序列一致，該模型顯示出如上文所述相同的表位及互補位。

實例7：抗FX/抗FIX(A) V_HH多肽的SPR分析

【0332】 此研究之目的為藉由表面電漿子共振(SPR)分析來評估所選擇之抗FX/抗FIX(a) V_HH多肽化合物與人類血漿衍生的FX及Benefix®的結合常數。

【0333】 通過SPR探測所純化的抗FX/FIX(a) V_HH多肽化合物與人類血漿衍生的FX (Haematologic Technologies股份有限公司，USA)的結合。

【0334】 簡言之，使用標準胺偶聯化學法在pH 5下將上述重組製備的抗GLA-FX固定在CM4感測晶片或Xantec HLC200M上。以10 μL/min的流速注入10 nM FX (Haematologic Technologies, USA)持續30秒。隨後根據下表9，以50 μL/min的流速注入1000、100、10、1、0.1及0 nM V_HH多肽化合物持續200秒，以使其與FX結合，隨後注射10分鐘運行緩衝液（10 mM HEPES、150 mM NaCl、5 mM $CaCl_2$、0.05%(v/v)介面活性劑P20、1 mg/mL牛血清白蛋白，pH 7.4），以可從FX上解離。該運行緩衝液也用於稀釋抗FX V_HH多肽化合物。使用由50 mM EDTA所組成的再生緩衝液於運行緩衝液中以30秒接觸時間及30 μL/min流速來實現晶片的再生。在25°C下收集結合資料，並使用由製造商(Biacore AB, Uppsala，或Bruker Analyser)提供的BiaEvaluation 4.1，根據1:1模型進行分析。

【0335】 在所有情況下，結合傳感圖均顯示出快速締合及快速解離的結合動力學曲線，從而排除了基於動力學分析的K_D判定。因此，所報告的K_D值是根據穩態分析判定的。分析產生了在下表9中所報告的結合常數。

【0336】 通過SPR探測所純化的抗FX/FIX(a) V_HH多肽化合物與Benefix®(Pfizer股份有限公司，USA)的結合。簡言之，使用標準胺偶聯化學法在pH 5下將上述重組製備的抗GLA-FIX固定在CM4感測晶片或Xantec HLC200M上。以10 μL/min的流速注入FIX (10 nM)持續1分鐘。隨後以30 μL/min的流速注入1000、100、10、1、0.1及0 nM V_HH多肽化合物持續4分鐘，以使其與FIX (Benefix®)結合，隨後注射5分鐘運行緩衝液（10 mM HEPES、150 mM NaCl、5 mM $CaCl_2$、0.05%(v/v)介面活性劑P20、1 mg/mL牛血清白蛋白，pH 7.4），以可從FIX上解離。該運行緩衝液也用於稀釋抗FIX V_HH多肽化合物。使用由50 mM EDTA所組成的再生緩衝液於運行緩衝液中以30秒接觸時間及30 μL/min流速來實現晶片的再生。在25°C下收集結合資料，並使用由製造商(Biacore AB, Uppsala)提供的BiaEvaluation 4.1，根據1:1模型進行分析。

【0337】 在所有情況下，結合傳感圖均顯示出快速締合及快速解離的結合動力學曲線，從而排除了基於動力學分析的K_D判定。因此，所報告的K_D值是根據穩態分析判定的。

【0338】 一系列化合物之結合常數分析係報告在表9中。FIX與所有化合物的結合親和力通常為低nM K_D，但有一些化合物顯示出其與FX的結合為高nM K_D。因此期望FX能沒有靶標介導的藥物傾向，可是會期望有FIX交互作用但僅限於所列化合物所需的穩態血漿濃度。因此，考慮到所列化合物的高效力，所列化合物能得到有意義的止血覆蓋範圍之期望所需血漿濃度預計為低nM濃度。

表9：所選擇之V_HH多肽化合物之估計結合常數

【0339】 由SPR分析判定之所選抗FX/抗FIX(a) V_HH多肽化合物與人類血漿衍生的FX及Benefix®之基於穩定態分析的估計結合常數。

化合物 ID	FX 結合子 K_D (nM)	FIX 結合子 K_D (nM)
化合物#4	229	11.7
化合物#5	199	2.7
化合物#6	232	4.8
化合物#7	82	2.3
化合物#8	96	2.5
化合物#9	70	1.1
化合物#10	190	5.5
化合物#11	42	6.4
化合物#12	26	7.8
化合物#13	300	2.0
化合物#14	42	1.6
化合物#15	350	3.0

實例8：抗FX/FIX(A) V_HH多肽在凝血酶生成試驗(TGT)測定法中的活性

【0340】 抗FX/FIX(a) V_HH多肽衍生物的促凝血活性係根據Hemker等人(Pathophysiol Haemost Thromb, 2002;32:249-253)描述的原理基於它們在促凝血合成磷脂膜的存在下促進凝血酶生成的能力來判定。包括了艾美賽珠單抗序列一致類似物(SIA)進行比較。使用補充有中和抗FVIII多株抗體的正常人類貧血小板血漿(NHP)(下文稱為HA-PPP)，在TGT測定法中測試每種V_HH多肽衍生物。

【0341】 在補充有綿羊抗人類FVIII多株抗體(pAb, Haematologic Technologies股份有限公司, VT, USA)的NHP(來自健康志願者)中之TGT係使用96孔盤螢光計(Fluoroscan Ascent FL，Thermolabsystems，赫爾辛基，芬蘭)通過標準校準的自動凝血曲線法(thrombography)進行。反應混合物係含有與0.1 μg/ml抗FVIII pAb預培養的36 μl NHP、4 μl測試化合物稀釋液(在20 mM HEPES、140 mM NaCl (pH7.4)、2% BSA中稀釋)、10 μl的1 pM組織因子(TF，pppLow，來自

Thrombinoscope BV，Maastricht，荷蘭)或1至8.3 U/ml人類因子Xia (Enzyme Research Laboratories, IN, USA)及10 μl FluCa受質(Thrombinoscope BV，Maastricht，荷蘭)。使用凝血酶校準物(Thrombinoscope BV，Maastricht，荷蘭)校準TGT測定，其中10 μl凝血酶校準物係與同0.1 μg/ml抗FVIII pAb預培養的36 μl NHP、4 μl緩衝液(20 mH HEPES，140 mM NaCl，pH 7.4，2%BSA)混合。大體上，TGT係於8種濃度的測試化合物(0.1、0.3、1、3、10、30、100及300nM，最終血漿濃度或類似濃度)或僅添加緩衝液(20 mM HEPES，140 mM NaCl，pH 7.4，2% BSA)(代表對照組)下進行。僅使用添加了NHP的緩衝液(20 mM HEPES，140 mM NaCl，pH 7.4，2% BSA)測量TGT中的正常對照組水平。使TGT進行總共60分鐘，並通過Thrombinoscope軟體(Thrombinoscope BV)分析TGT參數峰值凝血酶高度(nM)。見圖4中之所選試驗V_HH多肽衍生物(化合物#6)與比較物艾美賽珠單抗SIA及Mim8的代表滴定曲線。進行活性水平差異的評估以做為相對於比較物(例如艾美賽珠單抗SIA)的EC_{50}差異，或做為相對於艾美賽珠單抗SIA之最大凝血酶峰值高度下，給定V_HH多肽化合物之最大凝血酶峰值高度差異。後面的方法係用於篩選大量的V_HH多肽衍生物。

【0342】 為增強V_HH多肽衍生物的活性，鑑定出最佳的CDR突變組合以生成最大的活化效果。諸如實例5及6中所述之結晶結構模型係用以鑑定重要的互補位殘基，以及用於鑑定適於用半胱胺酸取代的表面暴露殘基以做為共軛位點，及鑑定降低PI的取代以增進生物可利用率。也調查了不同的連接子及不同的共軛類型。下表中概述了針對這些參數之一系列V_HH多肽衍生物的活性概況，且將個別描述。

【0343】 在表10中，其顯示出得自優化V_HH多肽衍生物之EC_{50}效價值係經活性增強突變優化。使用主要成分計算及隨機森林演算法進行突變組合以增強活性。V_HH多肽化合物#24為不具增強突變的初始衍生物，用以與此V_HH多肽衍

生物及艾美賽珠單抗SIA進行比較，在最佳的開發V_HH多肽衍生物中所得到的更佳效價分別增加到40倍及76倍。

表10：V_HH多肽衍生物經活性優化的TGT活性

化合物 ID	**延長部分 P 及 L_P** (延長部分的編號)	**pI**	**TGT 活性 EC_{50} 效價(nM)**
艾美賽珠單抗 SIA	-	-	113.9
Mim8	-	-	7.8
化合物#4	P3 及 L_P1 (1)	8.36	4.1
化合物#6	P2 及 L_P1 (2)	6.56	4.4
化合物#7	P2 及 L_P1 (2)	5.38	2.4
化合物#8	P2 及 L_P1 (2)	5.05	2.3
化合物#9	P2 及 L_P1 (2)	4.97	2.1
化合物#10	P2 及 L_P1 (2)	5.69	2.7
化合物#11	P2 及 L_P1 (2)	5.08	2.0
化合物#12	P2 及 L_P1 (2)	4.89	2.2
化合物#13	P2 及 L_P1 (2)	4.89	2.7
化合物#14	P2 及 L_P1 (2)	5.20	2.2
化合物#15	P2 及 L_P1 (2)	5.20	2.5
化合物#16	P2 及 L_P1 (2)	5.08	4.5
化合物#17	P2 及 L_P1 (2)	4.80	4.2
化合物#18	P2 及 L_P1 (2)	4.97	4.6
化合物#19	P2 及 L_P1 (2)	5.05	3.8
化合物#20	P2 及 L_P1 (2)	5.38	3.6
化合物#21	P2 及 L_P1 (2)	4.80	2.5
化合物#22	P2 及 L_P1 (2)	5.08	2.6
化合物#24	P3 及 L_P1 (1)	7.31	87.0
化合物#69	無	8.36	1.5

【0344】 在表11中，對V_HH多肽(CDR1-3之外)上的最佳共軛位點進行了篩選，其在用半胱胺酸取代（與經由連接子L_P1接附的C18二酸脂肪酸延長部分共軛或未共軛）之後保留了V_HH多肽的活性。

【0345】 總共測試95個導入Cys取代的表面暴露位點。使用連續編號的化合物#23/24做為參考物（代表V_HH多肽於CDR中不含任何增強突變），進行最佳共軛位點的篩選。測試了以下的殘基取代：G27C、V28C、V29C、Q30C、P31C、G32C、S34C、L35C、R36C、S38C、A40C、S42C、R55C、Q56C、A57C、P58C、G59C、K60C、E61C、R62C、Y77C、A78C、D79C、V81C、K82C、G83C、R84C、F85C、T86C、S88C、D90C、N91C、S92C、K93C、T95C、Y97C、Q99C、M100C、N101C、S102C、L103C、R104C、P105C、E106C、D107C、T108C、G156C、V157C、V158C、Q159C、P160C、G161C、G162C、S163C、L164C、R165C、S167C、A169C、S171C、R184C、Q185C、A186C、P187C、G188C、K189C、E190C、R191C、Y206C、A207C、D208C、S209C、V210C、K211C、G212C、R213C、F214C、T215C、S217C、D219C、N220C、S221C、K222C、T224C、Y226C、Q228C、M229C、N230C、S231C、L232C、R233C、P234C、E235C、D236C、T237C、及C端延伸導入Cys。

其中，以下41個V_HH多肽可使用HEK293表現系統來表現，並與包含有C18二酸延長子部分的試劑C3成功共軛：Q30C、P31C、Q56C、A57C、K60C、E61C、A78C、D79C、K82C、N91C、S92C、Q99C、R104C、G156C、V158C、Q159C、P160C、G161C、G162C、S163C、A169C、S171C、Q185C、A186C、P187C、G188C、K189C、E190C、A207C、D208C、S209C、V210C、T215C、S217C、D219C、N220C、S221C、K222C、S231C、R233C、及C端延伸導入Cys。這41個具有或不具有共軛的V_HH多肽係於TGT測定法中進行活係測試。該等具有或不具有共軛的V_HH多肽衍生物之相對活性水平係表示為V_HH多肽(衍生物)之最大凝血酶峰值高度相對於艾美賽珠單抗SIA之最大凝血酶峰值高度，並於表11中提出。因此，基於共軛前後所保留的活性，以下9個位點被鑑定為（例如使用C3試劑）導入游離Cys以與延長部分共軛的最佳位點：C端延伸導入Cys（化合物

#23/24)、Q30C (化合物#25/26)、V158C (化合物#27/28)、P160C (化合物#29/30)、G161C (化合物#31/32)、G188C (化合物#33/34)、E190C (化合物#35/36)、S209C (化合物#37/38)及S231C (化合物#39/40)。

表11：V_HH多肽衍生物之TGT活性、延長部分共軛位點篩選

V_HH 多肽衍生物(化合物#23/24 變體 w/o C-端延伸) Cys 共軛位點（連續編號）	**TGT 活性、最大峰值比率 (相對於艾美賽珠單抗 SIA)**
艾美賽珠單抗 SIA，參考對照	1.0
V_HH 多肽_1，Cys 於 C 端連接子延伸 SGGGGSGGGSCHHHHHH (SEQ ID NO: 23)中	1.4
V_HH 多肽_1，具 C18 二酸脂肪酸共軛	1.3
V_HH 多肽_2，Cys 取代 Q30C	1.8
V_HH 多肽_2，具 C18 二酸脂肪酸共軛	1.5
V_HH 多肽_3，Cys 取代 P31C	1.9
V_HH 多肽_3，具 C18 二酸脂肪酸共軛	1.0
V_HH 多肽_4，Cys 取代 E58C	1.9
V_HH 多肽_4，具 C18 二酸脂肪酸共軛	0.5
V_HH 多肽_5，Cys 取代 A78C	2.1
V_HH 多肽_5，具 C18 二酸脂肪酸共軛	0.3
V_HH 多肽_6，Cys 取代 K82C	1.7
V_HH 多肽_6，具 C18 二酸脂肪酸共軛	0.4
V_HH 多肽_7，Cys 取代 N91C	2.3
V_HH 多肽_7，具 C18 二酸脂肪酸共軛	0.9
V_HH 多肽_8，Cys 取代 S92C	2.3
V_HH 多肽_8，具 C18 二酸脂肪酸共軛	0.7
V_HH 多肽_9，Cys 取代 Q99C	2.1
V_HH 多肽_9，具 C18 二酸脂肪酸共軛	1.2
V_HH 多肽_10，Cys 取代 R104C	1.7
V_HH 多肽_10，具 C18 二酸脂肪酸共軛	0.6
V_HH 多肽_11，Cys 取代 G156C	0.1
V_HH 多肽_11，具 C18 二酸脂肪酸共軛	0.2
V_HH 多肽_12，Cys 取代 V158C	1.6
V_HH 多肽_12，具 C18 二酸脂肪酸共軛	1.4
V_HH 多肽_13，Cys 取代 Q159C	1.9
V_HH 多肽_13，具 C18 二酸脂肪酸共軛	1.4
V_HH 多肽_14，Cys 取代 P160C	2.0
V_HH 多肽_14，具 C18 二酸脂肪酸共軛	1.9
V_HH 多肽_15，Cys 取代 G161C	1.8
V_HH 多肽_15，具 C18 二酸脂肪酸共軛	1.6
V_HH 多肽_16，Cys 取代 G162C	1.9
V_HH 多肽_16，具 C18 二酸脂肪酸共軛	1.3
V_HH 多肽_17，Cys 取代 S163C	1.8

V_HH 多肽_17，具 C18 二酸脂肪酸共軛	1.4
V_HH 多肽_18，Cys 取代 A169C	2.0
VHV$_H$H H 多肽_18，具 C18 二酸脂肪酸共軛	1.0
V_HH 多肽_19，Cys 取代 S171C	2.1
V_HH 多肽_19，具 C18 二酸脂肪酸共軛	0.8
V_HH 多肽_20，Cys 取代 Q185C	1.1
V_HH 多肽_20，具 C18 二酸脂肪酸共軛	0.3
V_HH 多肽_21，Cys 取代 A186C	1.0
V_HH 多肽_21，具 C18 二酸脂肪酸共軛	0.4
V_HH 多肽_22，Cys 取代 P187C	2.2
V_HH 多肽_22，具 C18 二酸脂肪酸共軛	1.6
V_HH 多肽_23，Cys 取代 G188C	2.0
V_HH 多肽_23，具 C18 二酸脂肪酸共軛	1.8
V_HH 多肽_24，Cys 取代 K189C	1.7
V_HH 多肽_24，具 C18 二酸脂肪酸共軛	0.4
V_HH 多肽_25，Cys 取代 E190C	1.8
V_HH 多肽_25，具 C18 二酸脂肪酸共軛	1.5
V_HH 多肽_26，Cys 取代 A207C	1.9
V_HH 多肽_26，具 C18 二酸脂肪酸共軛	1.4
V_HH 多肽_27，Cys 取代 D208C	2.3
V_HH 多肽_27，具 C18 二酸脂肪酸共軛	1.5
V_HH 多肽_28，Cys 取代 S209C	1.9
V_HH 多肽_28，具 C18 二酸脂肪酸共軛	1.7
V_HH 多肽_29，Cys 取代 V210C	1.9
V_HH 多肽_29，具 C18 二酸脂肪酸共軛	1.5
V_HH 多肽_30，Cys 取代 T215C	1.9
V_HH 多肽_30，具 C18 二酸脂肪酸共軛	1.2
V_HH 多肽_31，Cys 取代 S217C	1.8
V_HH 多肽_31，具 C18 二酸脂肪酸共軛	1.2
V_HH 多肽_32，Cys 取代 D219C	1.8
V_HH 多肽_32，具 C18 二酸脂肪酸共軛	1.0
V_HH 多肽_33，Cys 取代 N220C	2.1
V_HH 多肽_33，具 C18 二酸脂肪酸共軛	0.8
V_HH 多肽_34，Cys 取代 S221C	1.9
V_HH 多肽_34，具 C18 二酸脂肪酸共軛	0.7
V_HH 多肽_35，Cys 取代 K222C	1.9
V_HH 多肽_35，具 C18 二酸脂肪酸共軛	1.1
V_HH 多肽_36，Cys 取代 S231C	1.9
V_HH 多肽_36，具 C18 二酸脂肪酸共軛	1.9
V_HH 多肽_37，Cys 取代 R233C	1.9
V_HH 多肽_37，具 C18 二酸脂肪酸共軛	1.4
V_HH 多肽_38，Cys 取代 Q56C	1.1
V_HH 多肽_38，具 C18 二酸脂肪酸共軛	0.3
V_HH 多肽_39，Cys 取代 A57C	0.9

V_HH 多肽_39，具 C18 二酸脂肪酸共軛	0.4
V_HH 多肽_40，Cys 取代 K60C	1.7
V_HH 多肽_40，具 C18 二酸脂肪酸共軛	0.4
V_HH 多肽_41，Cys 取代 D79C	1.7
V_HH 多肽_41，具 C18 二酸脂肪酸共軛	0.7

【0346】 對重組融合於兩個抗FX與抗FIX V_HH片段之間的不同連接子L_{1-2}進行測試以優化組合物（見表12）。該等V_HH多肽之相對活性水平係表示為V_HH多肽之最大凝血酶峰值高度相對於艾美賽珠單抗SIA之最大凝血酶峰值高度，並於表12中提出。從這些數據中，具有疏水性組成物連接子的V_HH多肽通常顯現出最高的相對活性，而具有酸性或親水性組成物連接子的V_HH多肽則顯現出最低的相對活性。具有由QAPGQA、GQAPGQ或類似物所組成之混合組成物連接子的V_HH多肽衍生物係顯示出中等的相對活性。最佳的連接子組成物較佳地會皆含有親水性及疏水性胺基酸，因此(例如)使用MS分析進一步調查該QAPGQA連接子的化學穩定性，其在等滲透緩衝液及中性pH中於37ºC的加速應力條件下顯示出兩週的高穩定性。

【0347】 因此，「40%至60%」至「60%至40%」之疏水性胺基酸與親水性胺基酸的比率是較佳的。所指出的V_HH多肽皆包括同一組的活性增強CDR突變，但CDR之外為不同組之PI降低的突變。

表12：具有不同連接子L_{1-2}特性之V_HH多肽序列的TGT活性

V_HH 多肽 ID	**連接子(L_{1-2})序列**	**TGT 活性、最大峰值比率** (相對於艾美賽珠單抗 SIA)
艾美賽珠單抗 SIA		1.0
化合物#41	QAPGQA	5.2
化合物#42	GIGIGIGIGI	5.5
化合物#43	GTGTGTGTGT	3.7
化合物#44	GVGVGVGVGV	5.7
化合物#45	GQAPGQ	4.9
化合物#46	GGGGSGGGS	5.0
化合物#47	QAPGQA	5.4
化合物#48	GLGLGLGLGL	7.0
化合物#49	GTGTGTGTGT	4.4

化合物#50	GVGVGVGVGV	5.6
化合物#51	GGGGSGGGS	4.1
化合物#52	QAPGQA	4.9
化合物#53	GEGEGEGEGE	3.1
化合物#54	GLGLGLGLGL	5.6
化合物#55	GTGTGTGTGT	4.9
化合物#56	GVGVGVGVGV	5.6
化合物#57	GQAPGQ	6.9

實例9：適合口服給藥劑型之具有SNAC及NAM的之抗FX/抗FIX(A) V_HH多肽衍生物製劑

【0348】 為了製備使用以N-(8-(2-羥基苯甲醯基)胺基)辛酸鈉(SNAC)及菸鹼醯胺(NAM)作為賦形劑的液體製劑進行口服研究，遵循以下程序。將SNAC稱重以得到濃度200 mg/ml，將NAM稱重以得到濃度1 M，將HEPES緩衝液稱重以得到最終濃度5 mM。將粉末轉移至玻璃瓶中並相應添加MilliQ-H_2O。用磁性攪拌棒攪拌溶液直到SNAC、NAM及硬脂酸鎂溶解。測量pH並用2 M NaOH調整至pH 8。因此，將2-5 mg/ml之經純化共軛V_HH多肽衍生物分批與200 mg/ml SNAC及最終濃度1 M的NAM進行液體配製。

【0349】 為了製備使用含SNAC及NAM的錠劑進行口服研究，遵循以下程序。將具有或不具有脂肪酸延長子共軛的純化V_HH多肽(衍生物)分批進行緩衝液交換至MilliQ-H2O中且使用0.1 M NaOH或0.1 M甲酸調整pH至8.0。溶解於MilliQ-H_2O中的V_HH多肽(衍生物)之濃度為0.5至4 mg/ml。使用迷你噴霧乾燥機B290 (BUCHI)，泵浦設定為5-6，進料流量為2 ml/min，入口溫度為 80-85°C，出口溫度為 45-50°C，噴嘴為1.5 mm且吸入量為 100%，對V_HH多肽(衍生物)進行噴霧乾燥。

【0350】 為了製備錠劑，將粉末混合以得到所要組成物。製備以下錠劑製劑。

製劑#1

V_HH多肽(衍生物)： 10-20 mg

SNAC: 300 mg

NAM: 0 mg

硬脂酸鎂： 5.5 mg

製劑#2

V_HH多肽衍生物： 10-20 mg

SNAC: 300 mg

NAM: 200 mg

硬脂酸鎂： 5.5 mg

製劑#3

V_HH多肽衍生物： 20 mg

SNAC: 150 mg

NAM: 100 mg

硬脂酸鎂： 1.25 mg

【0351】 將混合的粉末稱重，並使用Kilian Style One (Romaco)進行沖孔製成均質錠劑，沖孔設置成模擬旋轉式打錠機。

實例10：在大鼠中使用具不同PI變化突變之抗FX/抗FIX(A) V_HH多肽衍生物之口服及靜脈藥物動力學研究

【0352】 此研究之目的為調查抗FX/抗FIX(a) V_HH多肽衍生物中之pI變化突變對於藥物動力學參數（諸如口服生物可利用率）的影響。

【0353】 抗FX/FIX(a) V_HH多肽衍生物之液體製劑係靜脈給藥(IV) (IV製劑：20 mM Hepes, 150 mM NaCl, pH 7.4)或是根據實例9製備並通過口服胃管灌食法對平行組的Sprague Dawley大鼠進行給藥，這些大鼠在研究前至少在室內馴養了一周，並飼養在組籠中，可以隨意獲得標準的食物及水。口服給藥的大鼠從給藥前一天大約下午2點開始禁食，可以自由飲水，關在格子底的籠子裡（總共18小時）。給藥當天，所有大鼠在手術室裡適應了30分鐘。

【0354】 在給藥前立即採集血液樣本，隨後在給藥後的一些不同時間點採集血液樣本。口服(PO)給藥後的動物在格子底的籠子裡保持禁食，直到給藥後4小時。V_HH多肽衍生物的血漿濃度係使用基於γ-Glu的免疫測定法，其中使用了針對試驗分子延長部分之抗V_HH抗體(Novo Nordisk, Denmark)及抗γ-Glu的抗體(Novo Nordisk, Denmark)。在這裡，將96孔MaxiSorp孔盤(Nunc, 439454)塗覆2 μg/ml 抗γ-Glu抗體，使用PBS; 0.05% tween20; 1% BSA; pH 7.4洗滌及阻斷。洗滌步驟後，將在1%大鼠EDTA血漿中的化合物特異性校準物(0、2.7、8.2、24.7、74、222、667、2000 pM)和在最小稀釋度為100倍的大鼠EDTA血漿樣本在孔盤上培養，經由其γ-Glu延長部分捕獲V_HH多肽衍生物。在一額外的洗滌步驟後，將一自力生物素化的V_HH特異性抗體添加至孔盤上(0.5 nM)以組成三明治ELISA。在最後的洗滌步驟後，將辣根過氧化物酶(HRP)-鏈黴親合素作為檢測試劑加入到孔盤中。在加入顯色受質（如TMB (3,3',5,5'-四甲基聯苯胺)）後，檢測與V_HH多肽衍生物結合的生物素標記抗體量。使用光譜儀（例如SpectraMax® M2光譜儀(Molecular Devices)）測量光密度。反應係與過氧化物酶的濃度成正比，而過氧化物酶又與V_HH多肽衍生物的濃度成正比。基於這些暴露資料，使用Phoenix WinNonlin或開放原始碼的統計分析軟體R (套裝'NonCompart')計算非隔室藥物動力學參數。

【0355】 導入於CDR以外之表面暴露殘基上之下列降低pI的取代T28D、

K43Q、K65Q、N84D、R148Q、N159D、K172Q、K194Q及N213D（使用化合物#4作為模板進行連續編號）係於不同組合中進行測試（導致V_HH多肽衍生物化合物#59為化合物#64及化合物#5），並與未調整pI之V_HH多肽衍生物化合物#4進行比較。

【0356】 下表13及14給出了V_HH衍生物的結果，且顯示降低pI對口服生物可利用率的影響。

【0357】 將pI從大約8.36降低到5.85會增加生物可利用率5到10倍（見表13）。沒有觀察到降低pI對半衰期的影響（見表14）。

表13：具有PO大鼠模型中所試驗之工程改造pI突變的V_HH多肽衍生物的口服生物可利用率數據

衍生物 ID	延長子，共軛位點	pI	N (PO 群)	PO 劑量 (nmol/kg)	PO 口服生物可利用率 (%)
化合物#4	C18 二酸，C 端 Cys 共軛	8.36	6	338	0.02
化合物#59	C18 二酸，C 端 Cys 共軛	6.57	7	214-230	0.04
化合物#5	C18 二酸，C 端 Cys 共軛	6.56	7	215-233	0.07
化合物#60	C18 二酸，C 端 Cys 共軛	6.42	7	166-167	0.14
化合物#61	C18 二酸，C 端 Cys 共軛	6.42	7	166-167	0.10
化合物#62	C18 二酸，C 端 Cys 共軛	6.09	7	190-208	0.19
化合物#63	C18 二酸，C 端 Cys 共軛	6.09	7	166-167	0.15
化合物#64	C18 二酸，C 端 Cys 共軛	5.85	6	338	0.12

表14：具有IV大鼠模型中所試驗之工程改造pI突變的V_HH多肽衍生物的PK數據

衍生物 ID	延長子，共軛位點	pI	N (IV 群)	IV 劑量 (nmol/kg)	IV MRTHL* (天數)
化合物#4	C18 二酸，C 端 Cys 共軛	8.36	3	6.7	0.51
化合物#59	C18 二酸，C 端 Cys 共軛	6.57	3	6.6	0.51
化合物#5	C18 二酸，C 端 Cys 共軛	6.56	3	6.6	0.53
化合物#60	C18 二酸，C 端 Cys 共軛	6.42	3	6.6	0.57
化合物#61	C18 二酸，C 端 Cys 共軛	6.42	3	6.6	0.57

化合物#62	C18 二酸，C 端 Cys 共軛	6.09	3	6.6	0.72
化合物#63	C18 二酸，C 端 Cys 共軛	6.09	3	6.6	0.43
化合物#64	C18 二酸，C 端 Cys 共軛	5.85	3	6.7	0.48

* MRTHL：平均停留時間終末半衰期。MRTHL的計算方法是MRT*ln(2)。

實例11：在大鼠中的靜脈藥物動力學研究：具不同的延長子之抗FX/抗FIX(A) V_HH多肽(衍生物)

【0358】 此研究之目的為調查不同的延長子對於V_HH多肽衍生物之藥物動力學參數（諸如半衰期）的影響。

【0359】 抗FX/FIX(a) V_HH多肽衍生物之液體製劑係根據實例9製備並通過靜脈(IV)對平行組的Sprague Dawley大鼠進行給藥；這些大鼠在研究前至少在室內馴養了一周，並飼養在組籠中，可以隨意獲得標準的食物及水。口服給藥的大鼠從給藥前一天大約下午2點開始禁食，可以自由飲水，關在格子底的籠子裡（總共18小時）。給藥當天，所有大鼠在手術室裡適應了30分鐘。在給藥前立即採集血液樣本，隨後在給藥後的一些不同時間點採集血液樣本。V_HH多肽(衍生物)的血漿濃度係使用基於His標籤或γ-Glu的免疫測定法測量，其中使用了針對V_HH多肽衍生物之抗V_HH抗體(Novo Nordisk)以及針對融合至該試驗分子之His標籤的抗His標籤的抗體(R&D systems, MAB050)或是針對試驗分子之延長部分的抗γ-Glu (Novo Nordisk, Denmark)。這兩種免疫測定法設置具有相同的靈敏度，且在V_HH多肽化合物沒有融合至該分子之His標籤的時候使用後者。將96孔MaxiSorp孔盤(Nunc, 439454)塗覆His標籤或抗γ-Glu抗體，使用PBS; 0.05% tween20; 1% BSA; pH 7.4洗滌及阻斷。洗滌步驟後，將在1%大鼠EDTA血漿中的衍生物特異性校準物(0、2.7、8.2、24.7、74、222、667、2000 pM)和在最小稀釋度為100倍的大鼠EDTA血漿樣本在孔盤上培養，經由其His標籤或γ-Glu延長部分捕獲V_HH多肽衍生物。在一額外的洗滌步驟後，將一自力生物素化的V_HH特異性抗體添加至孔盤上(0.5 nM)以組成三明治ELISA。在最後的洗滌步驟後，將辣根過氧化物酶(HRP)-鏈黴親合素作為檢測試劑加入到孔盤中。在加入顯色受質（如TMB (3,3',5,5'-四甲基聯苯胺)）後，檢測與V_HH多肽衍生物結合的生物素標記抗體量。使用光譜儀（例如SpectraMax® M2光譜儀(Molecular Devices)）測量光密

度讀數。反應係與過氧化物酶的濃度成正比，而過氧化物酶又與V_HH多肽衍生物的濃度成正比。基於這些暴露資料，使用Phoenix WinNonlin或開放原始碼的統計分析軟體R (套裝'NonCompart')計算非隔室藥物動力學參數。

【0360】 下表15給出了具有不同延長子類型之V_HH多肽衍生物的結果。結果顯示了使用不同的脂肪酸及白蛋白結合肽作為延長子對中位停留時間終末半衰期(MRTHL)的影響。

【0361】 與未延長的多肽相比，對於具有C18、C20及四唑脂肪酸側鏈共軛的V_HH多肽衍生物（化合物#66、化合物#67及化合物#68：增加3至4倍），以及具有白蛋白結合肽延長子融合的V_HH多肽衍生物（化合物#70、化合物#71、化合物#72及化合物#73：增加1.9至3.6倍），在白蛋白結合肽與V_HH多肽之間使用長度30個殘基的GlySer L_P連接子融合在C端有最佳效果。具有短C12脂肪酸的V_HH多肽衍生物化合物#65顯示出最低的半衰期。這表明，為了得到V_HH多肽衍生物的最佳延長期，為了半衰期循環的延長，長於C12的脂肪酸是較佳的（諸如(例如)C16、C18或C20）。

表15：具有IV大鼠模型中所試驗之不同延長子的V_HH多肽的PK數據

衍生物 ID	延長子及共軛位點	pI	N (IV 群)	IV 劑量(nmol/kg)	IV MRTHL* (天數)
化合物#64	無延長子	7.11	5	6.5	0.19
化合物#65	C12 二酸，C 端 Cys 共軛	7.74	5	6.6	0.11
化合物#66	C18 二酸，C 端 Cys 共軛	7.74	5	6.6	0.63
化合物#67	C20 二酸，C 端 Cys 共軛	7.74	5	6.6	0.75
化合物#68	四唑二酸，C 端 Cys 共軛	7.74	5	6.6	0.71
化合物#69	無延長子	8.36	3	6.9	0.13
化合物#4	C18 二酸，C 端 Cys 共軛	8.36	3	6.7	0.51
化合物#70	白蛋白結合肽，10-GlySer 連接子，N 端融合	7.11	2	6.7	0.36
化合物#71	白蛋白結合肽，30-GlySer 連接子，N 端融合	7.11	2	6.7	0.34
化合物#72	白蛋白結合肽，10-GlySer 連接子，C 端融合	7.18	2	6.7	0.36
化合物#73	白蛋白結合肽，30-GlySer 連接子，C 端融合	7.18	2	6.7	0.70

* MRTHL：平均停留時間終末半衰期。MRTHL的計算方法是MRT*ln(2)。

實例12：在狗中的口服及靜脈藥物動力學研究：與SNAC及NAM一起配製的抗FX/抗FIX(A) V_HH多肽(衍生物)

【0362】 此研究之目的為調查脂肪酸延長子對半衰期的影響，且是否降低pI會使口服生物可利用率增進。

【0363】 適合的抗FX/FIX(a) V_HH多肽(衍生物)製劑係分別被靜脈注射給藥或製備成錠劑並向米格魯狗群口服給藥。狗在隔夜禁食後於早上投藥，在單次給藥後保持禁食3-5小時。在口服研究的一個子集§中，在口服給藥前10分鐘給狗皮下注射了大約3 nmol/kg的升糖素。在給藥前立即採集血液樣本，隨後在給藥後的一些不同時間點採集血液樣本。V_HH多肽的血漿濃度係使用基於His標籤或γ-Glu-連接子的免疫測定法測量，其中使用了針對V_HH多肽之抗V_HH抗體(Novo Nordisk, Denmark)以及針對融合至該試驗分子之His標籤的抗His標籤的抗體(R&D systems, MAB050)或針對該試驗分子之延長部分的抗γ-Glu (Novo Nordisk, Denmark)。這兩種免疫測定法設置有相近的靈敏度，且在V_HH多肽化合物沒有融合至該分子的His標籤時使用後者。將96孔MaxiSorp孔盤(Nunc, 439454)塗覆抗體，使用PBS; 0.05% tween20; 1% BSA; pH 7.4洗滌及阻斷。洗滌步驟後，將在1%狗EDTA血漿中的化合物特異性校準物(0、2.7、8.2、24.7、74、222、667、2000 pM)和在最小稀釋度為100倍的狗EDTA血漿樣本在孔盤上培養，經由其His Tag或γ-Glu連接子模體捕獲V_HH多肽化合物。在一額外的洗滌步驟後，將一自力生物素化的V_HH特異性抗體添加至孔盤上(0.5 nM)以組成三明治ELISA。在最後的洗滌步驟後，將辣根過氧化物酶(HRP)-鏈黴親合素作為檢測試劑加入到孔盤中。在加入顯色受質（如TMB (3,3',5,5'-四甲基聯苯胺)）後，檢測與V_HH多肽化合物結合的生物素標記抗體量。使用光譜儀（例如SpectraMax® M2光譜儀(Molecular Devices)）測量光密度讀數。反應係與過氧化物酶的濃度成正比，而過氧化物酶又與V_HH多肽化合物的濃度成正比。基於這些暴露資料，使用Phoenix WinNonlin

或開放原始碼的統計分析軟體R (套裝'NonCompart')計算非隔室藥物動力學參數。

§ 表示在口服給藥前10分鐘，皮下注射大約3 nmol/kg的升糖素。

【0364】 下表16及17給出了三種C18二酸共軛的V_HH多肽衍生物的結果，且確認了

a)脂肪酸延長子對半衰期的影響，及

b)降低pI會使口服生物可利用率增進。

【0365】 因此，當V_HH多肽衍生物與所述脂肪酸共軛時，與未延長的化合物相比，在狗中觀察到的半衰期增加了370至710倍。該在C端連接子具有兩個C16二酸脂肪酸共軛之化合物化合物#6顯示出半衰期增長最高，大約為7.6天。

【0366】 在狗中，SNAC:NAM製劑比僅SNAC製劑增加了口服生物可利用率2至8倍。因此，在口服製劑中添加NAM有明顯的效果，使口服生物可利用率達到或超過臨床相關的水準，即大約0.1%。

【0367】 總體來說，考慮到效價、平均停留時間及口服生物可利用率，會期望本文所揭露之V_HH多肽衍生物在口服投予時能有益於具有或沒有抑制子之A型血友病以及後天性A型血友病的治療。

表16：具有狗模型中所試驗之工程改造pI突變及脂肪酸側鏈共軛的V_HH多肽(衍生物)的口服生物可利用率數據

衍生物 ID	延長子，共軛位點	pI	N (PO 群)	PO 製劑	PO 劑量 (nmol/kg)	口服生物可利用率
化合物#4	C18 二酸，C 端 Cys 共軛	8.36	7	「製劑#1」	83-216	0.02
化合物#74§	無延長子	7.74	6	「製劑#1」	86	0.05
化合物#61	C18 二酸，C 端 Cys 共軛	6.09	7	「製劑#2」	114-225	0.16
化合物#63	C18 二酸，C 端 Cys 共軛	5.85	7	「製劑#2」	132-249	0.13
化合物#6§	x2 C16 二酸，C 端 Cys 共軛	5.61	5	「製劑#2」	99-152	0.09
化合物#15§	x2 C16 二酸，C 端 Cys 共軛	4.89	6	「製劑#3」	54-71	0.24

表17：具有IV狗模型中所試驗之工程改造pI突變及脂肪酸側鏈共軛的V_HH多肽的PK數據

衍生物 ID	延長子，共軛位點	pI	N (IV 群)	IV 劑量 (nmol/kg)	IV MRTHL* (天數)
化合物#4	C18 二酸，C 端 Cys 共軛	8.36	2	6.5-6.9	3.7
化合物#74	無延長子	7.74	3	1.9-2.0	< 0.01
化合物#61	C18 二酸，C 端 Cys 共軛	6.09	2	6.5-6.6	4.5
化合物#63	C18 二酸，C 端 Cys 共軛	5.85	2	6.6-6.7	4.5
化合物#6	x2 C16 二酸，C 端 Cys 共軛	5.61	3	1.7-2.3	7.1
化合物#15	x2 C16 二酸，C 端 Cys 共軛	4.89	2	2.6	4.1

* MRTHL：平均停留時間終末半衰期。MRTHL的計算方法是MRT*ln(2)。

實例13：在豬中的靜脈藥物動力學研究：與C18共軛的抗FX/抗FIX(A) V_HH多肽(衍生物)

【0368】 此研究之目的為調查具有及不具有C18二酸延長子基團之V_HH多肽(衍生物)的藥物動力學影響。

【0369】 將適合的抗FX/FIX(a) V_HH多肽(衍生物)製劑係分別向迷你豬群(*Sus scrofa domesticus*)靜脈注射給藥及皮下給藥。在給藥前立即採集血液樣本，隨後在給藥後的一些不同時間點採集血液樣本。V_HH多肽(衍生物)的血漿濃度係使用基於His標籤或基於γ-Glu的免疫測定法測量，其中使用了針對V_HH多肽(衍生物)之抗V_HH抗體(Novo Nordisk)以及針對融合至該試驗分子之His標籤的抗His標籤的抗體(R&D systems, MAB050)或是針對試驗分子之延長部分的抗γ-Glu (Novo Nordisk, Denmark)。這兩種免疫測定法設置具有相同的靈敏度，且在V_HH多肽化合物沒有融合至該分子之His標籤的時候使用後者。將96孔MaxiSorp孔盤(Nunc, 439454)塗覆His標籤或抗γ-Glu抗體，使用PBS; 0.05% tween20; 1% BSA; pH 7.4洗滌及阻斷。洗滌步驟後，將在1%豬EDTA血漿中的衍生物特異性校準物(0、2.7、8.2、24.7、74、222、667、2000 pM)和在最小稀釋度為100倍的豬EDTA血漿樣本在孔盤上培養，經由其His標籤或γ-Glu延長部分捕獲V_HH多肽化合物。在一額外的洗滌步驟後，將一自力生物素化的V_HH特異性抗體添加至孔盤上(0.5 nM)以組成三明治ELISA。在最後的洗滌步驟後，將辣根過氧化物酶(HRP)-鏈黴親合素作為檢測試劑加入到孔盤中。在加入顯色受質（如TMB (3,3',5,5'-四甲基聯苯胺)）後，檢測與V_HH多肽衍生物結合的生物素標記抗體量。使用光譜儀（例如SpectraMax® M2光譜儀(Molecular Devices)）測量光密度讀數。反應係與過氧化物酶的濃度成正比，而過氧化物酶又與V_HH多肽衍生物的濃度成正比。使用開放原始碼的統計分析軟體R (套裝'NonCompart')計算非隔室藥物動力學參數。具有或不具有脂肪酸延長子之V_HH多肽的結果係顯示於下表18，確認了脂肪酸延長子

共軛對半衰期延長的影響，如於實例11、12及13中所觀察到。

【0370】 因此，當V_HH多肽與所述脂肪酸C18二酸延長子共軛時，與未延長的V_HH化合物相比，在豬中觀察到的半衰期增加了大約15至20倍。

表18：具有豬模型中所試驗之工程改造pI突變及脂肪酸延長子共軛的V_HH多肽的PK數據

衍生物 ID	**延長子，共軛位點**	**pI**	**N** (IV 或 SC 群)	**劑量** (nmol/kg)	**IV MRTHL*** (天數)
化合物#75	無延長子	8.95	3 (SC)	30	0.59
化合物#75	無延長子	8.95	2 (IV)	30	0.24
化合物#4	C18 二酸，C 端 Cys	8.36	3 (IV)	5.3	3.6
化合物#63	C18 二酸，C 端 Cys	5.85	3 (IV)	4.8	4.7

* MRTHL：平均停留時間終末半衰期。MRTHL的計算方法是MRT*ln(2)。

實例14：經優化抗FX/抗FIX(A)多肽衍生物在小鼠尾靜脈橫斷(TVT)出血模型中的藥物動力學效用

【0371】 在中度出血之HA小鼠模型中測試活體內功效。在TVT模型中，先前已證實投予FVIII會降低出血至野生型小鼠上觀察到的相同程度。為克服所測化合物缺少小鼠跨物種反應性，HA小鼠係於出血實驗之前補充人類FIX及FX。出血期結束時，藉由分光光度法測量血紅蛋素來判定總失血量。所指出的V_HH多肽衍生物及FIX的血漿水平係藉由發光氧通道測定法分別使用抗V_HH (Novo Nordisk, Denmark)及抗γGlu-連接子抗體(Novo Nordisk, Denmark)及抗FIX抗體(LS-B7226、LSBio及FIX-2F24，公司內部殖株)進行量化。FX水平係藉由市售FX ELISA (KSP134, Nordic Biosite)來定量。從所指出的V_HH多肽衍生物之劑量-反應研究中，藉由將數據擬合至具有共同平原值(> 0)之三參數逆對數(劑量)反應方程式來判定EC_{50}值。使用GraphPad Prism軟體(版本9.0.1)，採用1%的ROUT係數自動消除離群點，且平方和按（失血量）加權計算。

【0372】 與載劑組及野生型非血友病組相比，所測試的V_HH多肽衍生物之劑量反應研究顯示失血量明顯減少，分別達到野生型小鼠中觀察到的失血水平，如表19中所示。表20列出了EC_{50}有效劑量範圍的估計，因為3個所測化合物皆在低劑量(nmole /公斤)下觀察到50%的效果而顯示出高效價。

表19：抗FX/抗FIX(a)多肽衍生物在TVT出血小鼠模型中效用

衍生物# / 劑量 (μmole/kg)	**出血時間** (min, AVE ± SD, n = 6)	**失血量** (nmole, AVE ± SD, n = 6)
載劑	21.0 ± 3.5	4594 ± 1326
野生型，非血友病	5.4 ± 0.4	879 ± 438
化合物#4 0.003 μmole/kg 0.005 μmole/kg 0.006 μmole/kg 0.010 μmole/kg 0.013 μmole/kg	 25.8 ± 3.0 23.2 ± 6.7 19.7 ± 11.2 13.9 ± 8.7 11.0 ± 8.6	 6979 ± 1281 6204 ± 1496 5232 ± 3698 2973 ± 2973 2264 ± 2264
化合物#6		

0.003 μmole/kg 0.005 μmole/kg 0.006 μmole/kg 0.010 μmole/kg 0.013 μmole/kg	26.4 ± 4.4 18.4 ± 6.7 11.8 ± 9.6 19.2 ± 10.9 7.1 ± 4.3	7445 ± 1053 5143 ± 1861 3771 ± 3374 4532 ± 3277 1024 ± 1050
化合物#15 0.0065 μmole/kg 0.0065 μmole/kg 0.0097 μmole/kg 0.0129 μmole/kg	 9.5 ± 7.3 14.8 ± 8.3 7.0 ± 4.7 6.9 ± 1.7	 2408 ± 2391 4545 ± 2417 1223 ± 1163 1438 ± 798

表20：TVT出血小鼠模型之抗FX/抗FIX(a)多肽衍生物的EC_{50}估計

衍生物 ID	**出血時間 EC_{50}** (μmole/kg)	**出血量 EC_{50}** (μmole/kg)
化合物#4	0.0097	0.0094
化合物#6	0.0077	0.0087
化合物#15	0.0047	0.0060

實例15：經優化抗FX/抗FIX(A) V_HH多肽衍生物在口服給藥後的體外活性判定

【0373】 在實例12的野生型狗的藥物動力學(PK)研究中，為了界定化合物#6的PO給藥特點，在給藥後30分鐘及90分鐘兩個獨立時間點上測定顯色活性。將測得的FVIII模擬、顯色活性與化合物#6的血漿暴露量進行比較，以判定PO給藥後於血清中之活性V_HH多肽衍生物的分額。

【0374】 化合物#6的顯色活性係使用市售FVIII顯色活性測定(FVIII:C, Hyphen Biomed, France)來判定，以分析狗血清樣本。對於校準曲線，將化合物#6摻入10%的狗血清中，用同樣的測定法對血清樣本進行10倍稀釋分析。對10倍稀釋的最終活性結果進行10倍校正。藉由使用抗V_HH (Novo Nordisk，丹麥)及抗γGlu-連接子抗體(Novo Nordisk，丹麥)的發光氧通道(LOCI)測定，對化合物#6的血漿暴露水平進行量化。其數據係概述於表21中。在給藥後30分鐘及90分鐘，血清中可測得FVIII:C的顯色活性，基於顯色活性的化合物#6的定量係與基於使用

LOCI測定之化合物#6血漿暴露測量的估計相似。化合物#6定量方面的任何差異預測是因測定靈敏度的差異所引起。數據顯示出，PO投予的V_HH多肽衍生物在狗口服後具有完全的活性，表明製造口服的FVIII模擬藥物來治療患有例如A型血友病之患者的概念是可行的。

表21：抗FX/抗FIX(a)多肽衍生物在PO給藥後的體外活性

口服後的時間	動物	FVIII:C 顯色活性 (pM，血清)	化合物#6 暴露 (pM，血漿)	活性/暴露 比率%
30 分鐘	動物 1	633	414	153%
	動物 2	1740	905	192%
	動物 3	445	247	180%
	動物 4	1930	1490	129%
90 分鐘	動物 1	1640	1290	127%
	動物 2	3820	3130	122%
	動物 3	2470	2110	117%
	動物 4	7920	6550	121%

實例16：抗FX V_HH片段化合物#76之結晶及互補位/表位定位

【0375】 此研究之目的為判定命名為化合物#76 (VHH-1.15)之V_HH片段的表位及互補位殘基。

結晶化

【0376】 與實例4中所鑑定之對應於人類FX活化肽序列aa31-37之合成肽N-端-NPFDLLD-C-端(購自Apigenex)以1:4莫耳比率混合的V_HH片段化合物#76之結晶係使用坐滴蒸氣擴散技術生長。將250 nl的5.75 mg/ml複合物於20 mM Tris-HCl (pH 7.4)、50 mM NaCl中的蛋白質溶液與250 nl的4 % (v/v) Tacsimate (1.8305 M丙二酸、0.25 M檸檬酸三銨、0.12 M琥珀酸、0.3 M DL-蘋果酸、0.4 M三水合醋酸鈉、0.5 M甲酸鈉及0.16 M酒石酸二銨) (pH 5.0)、12 % (w/v)聚乙二醇3350 (作為沈澱劑)混合並以80 µl沈澱劑培養。在18 °C下培養5天後，將結晶化盤轉移到5 °C。在三個月內出現結晶。

繞射數據收集

【0377】 結晶係於在液態氮中急速冷卻之前於加有20%的乙二醇的沈澱劑中冷凍保護。繞射數據係以100K於Swiss Light Source光束線X10SA (1.0000 Å波長)使用來自Dectris的Eiger2 16M像素偵測器收集。數據之自動索引、集成及縮放係使用來自XDS套裝的程式執行（繞射數據統計係於表22中概述）。

結構判定及精修

【0378】 不對稱單元含有兩個化合物#76:FX AP 31-37複合物。結構係藉由分子置換測定，使用如於程式套裝Phenix中實施的Phaser，並使用一相關V_HH之預定結晶結構做為搜尋模型，見實例6。導入化合物#76的正確胺基酸序列，在差異電子密度定位中手動建立FX AP 31-37模型，且全使用COOT。在幾輪的Phenix精修及COOT手動重建期間，觀察到蘇胺酸殘基117上的O-醣基化及酪胺酸殘基109上的硫酸化並建模。精修統計可在表22中找到。

表22：數據收集及精修統計

波長(Å)	1
解析度範圍(Å)	41.89 - 1.7 (1.761 - 1.7)
空間群	P 4_1 2_1 2
晶格(Å, deg)	66.54 66.54 161.74 90 90 90
總反射數	534639 (54552)
獨立反射數	40883 (4000)
多重度	13.1 (13.6)
完整度(%)	99.59 (99.48)
平均訊雜比強度 I/σ(I)	17.03 (0.83)
Wilson B-因子($Å^2$)	35.51
合併的 R 因子	0.07098 (3.508)
非重複性合併的 R 因子	0.07398 (3.642)
精確指標合併的 R 因子	0.0206 (0.9736)
CC1/2	0.999 (0.72)
CC*	1 (0.915)
用於精修的反射數.	40750 (3981)
用於計算自由 R 因子的反射數	1993 (193)
工作 R 因子	0.1828 (0.3798)
自由 R 因子	0.2077 (0.4038)
CC(工作)	0.963 (0.862)

CC(自由)	0.949 (0.702)
非氫原子之數目.	2225
巨分子	1958
配體	84
溶劑	217
蛋白質殘基	254
RMS(鍵長)	0.010
RMS(鍵角)	1.15
拉氏圖(Ramachandran)偏好比例(%)	96.67
拉氏圖允許比例(%)	2.92
拉氏圖極端比例(%)	0.42
離群旋轉異構體(%)	0.50
對撞分數	3.59
平均 B-因子(Å^2)	47.24
巨分子	46.21
配體	71.05
溶劑	51.13
TLS 群數目	1

對於最高解析殼之統計係顯示於括弧中。

表位及互補位之判定

【0379】 根據對應之連續編號的FX活化肽序列(SEQ ID NO:2)，該合成肽的表位（其定義為特徵為在離V_HH多肽化合物#76中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的FX活化肽殘基）係包含以下來自合成肽N173、P174、F175、L177、L178及D179的殘基。

【0380】 V_HH片段化合物#76的互補位（其定義為特徵為在離合成肽中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的化合物#76殘基）係包含以下來自化合物#76的殘基：A33、M34、G35、W47、V48、A49、A50、I51、S52、S57、T58、N59、Y60、A61、A97、A98、D99、G105、L107及Y109 (SEQ ID NO:734)（連續編號）。

實例17：抗FX V_HH片段化合物#77之結晶及互補位/表位定位

【0381】 此研究之目的為判定命名為化合物#77 ($V_HH1.13$)之V_HH片段的互補位及表位殘基。

結晶化

【0382】 與對應於人類FX活化肽序列aa31-37之合成肽N-端-NPFDLLD-C-端(由Apigenex公司合成)以1:4莫耳比率混合之產自CHO的V_HH片段化合物#77之結晶係使用坐滴蒸氣擴散技術生長。將250 nl的6.1 mg/ml複合物於20 mM Tris-HCl (pH 7.4)、50 mM NaCl中的蛋白質溶液與250 nl的4 % (v/v) Tacsimate (1.8305 M丙二酸、0.25 M檸檬酸三銨、0.12 M琥珀酸、0.3 M DL-蘋果酸、0.4 M三水合醋酸鈉、0.5 M甲酸鈉及0.16 M酒石酸二銨) (pH 4.0)、11 % (w/v) PEG 3350 (作為沈澱劑)混合並以80 µl沈澱劑培養。在5 °C下培養4天出現結晶。

繞射數據收集

【0383】 結晶係於在液態氮中急速冷卻之前於加有20%的乙二醇的沈澱劑中冷凍保護。繞射數據係以100K於Swiss Light Source光束線X10SA (1.0000 Å波長)使用來自Dectris的Eiger2 16M像素偵測器收集。數據之自動索引、集成及縮放係使用來自XDS套裝的程式執行（繞射數據統計係於表23中概述）。

結構判定及精修

【0384】 不對稱單元含有兩個化合物#77:FX AP 31-37複合物。結構係藉由分子置換測定，使用如於程式套裝Phenix中實施的Phaser，並使用來自實例16之化合物#76:FX AP 31-37之結晶結構的完全不對稱單元做為搜尋模型。導入化合物#77的正確胺基酸序列，並進行幾輪的Phenix精修及COOT手動重建。精修統計可在表23中找到。

表23：數據收集及精修統計

波長(Å)	1.0000
解析度範圍(Å)	32.88 - 1.7 (1.761 - 1.7)

空間群	P 4_1 2_1 2
晶格(Å, deg)	58.99 58.99 160.25 90 90 90
總反射數	418459 (41888)
獨立反射數	31790 (3080)
多重度	13.2 (13.6)
完整度(%)	98.90 (97.90)
平均訊雜比強度 I/σ(I)	16.44 (1.79)
Wilson B-因子(Å^2)	26.81
合併的 R 因子	0.08787 (1.558)
非重複性合併的 R 因子	0.09151 (1.618)
精確指標合併的 R 因子	0.02521 (0.4322)
CC1/2	0.999 (0.762)
CC*	1 (0.93)
用於精修的反射數.	31765 (3079)
用於計算自由 R 因子的反射數	1588 (154)
工作 R 因子	0.1717 (0.2765)
自由 R 因子	0.2112 (0.3009)
CC(工作)	0.960 (0.903)
CC(自由)	0.935 (0.883)
非氫原子之數目	2253
巨分子	1971
配體	50
溶劑	232
蛋白質殘基	254
RMS(鍵長)	0.011
RMS(鍵角)	1.03
拉氏圖(Ramachandran)偏好比例(%)	98.33
拉氏圖允許比例(%)	1.67
拉氏圖極端比例(%)	0.00
離群旋轉異構體(%)	0.49
對撞分數	3.83
平均 B-因子(Å^2)	35.30
巨分子	33.83
配體	66.50
溶劑	41.00
TLS 群數目	1

對於最高解析殼之統計係顯示於括弧中。

表位及互補位之判定

【0385】 根據對應之連續編號的FX活化肽序列(SEQ ID NO:2)，該合成肽的表位（其定義為特徵為在離V_HH片段化合物#77中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的FX活化肽殘基）係包含以下來自合成肽N173、P174、F175、L177、L178及D179的殘基。

【0386】 V_HH片段化合物#77的互補位（其定義為特徵為在離合成肽中之重原子4.0 Å的距離內有一個重原子(即非氫原子)的化合物#77殘基）係包含以下來自化合物#77的殘基：

A33、M34、G35、W47、V48、A49、A50、I51、S52、S57、T58、N59、Y60、A61、A97、A98、D99、G105、L107、Y109 (SEQ ID NO:735)（連續編號）。

實例18：雙特異性V_HH多肽衍生物之穩定態Fxa生成動力學

【0387】 此研究之目的為基於抗FIXa/FX雙特異性V_HH多肽衍生物在促凝血磷脂膜的存在下之藉由FIXa促進FX活化的能力，來判定抗FIXa/FX雙特異性V_HH多肽衍生物之促凝血活性。所測試之化合物係列於表24中且包括了艾美賽珠單抗SIA進行比較。

【0388】 各化合物之穩定態Fxa生成活性係報告為在給定化合物濃度下的Michaelis-Menten動力學參數（Michaelis常數(K_M)和一階速率常數(k_{cat}/K_M)）。這些化合物的最終測定濃度係顯示於表24中，其係於測定緩衝液(50 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, 0.1% (w/v) PEG8000, pH 7.3 + 1 mg/ml BSA)中與0.1 nM人類血漿衍生的FIXa (Haematologic Technologies股份有限公司，USA)及20 μM 25:75的磷脂絲胺酸:磷脂醯膽鹼磷脂囊(Haematologic Technologies股份有限公司，USA)一起預培養5分鐘。接著從500 nM開始，加入兩倍連續稀釋之人類血

漿衍生的FX (Haematologic Technologies股份有限公司，USA)來開始進行活化。在室溫下活化7分鐘之後，添加25 µl淬滅緩衝液(50 mM HEPES, 100 mM NaCl, 60 mM EDTA, 0.1% PEG8000, pH 7.3 + 1 mg/ml BSA)使反應物(50 µl)淬滅。藉由添加25 µl 2 mM S-2765顯色受質(Chromogenix，瑞典)，並在微量盤讀取儀中測量在405 nm的吸光度(ΔOD/min)來測量顯色受質的轉化率，以測定所生成的Fxa數量。同樣地，在FIXa濃度為10 nM及反應時間為5至10分鐘下，判定受游離FIXa的FX活化。為了將在405 nm測得的吸光度轉換為相應的FXa濃度，在上述記錄405 nm的吸收度之前，在0-5 nM (50 µl)的FXa標準曲線中加入25 µl淬滅緩衝液及25 µl 2 mM S-2765顯色受質。以ΔOD/min作為FXa濃度的函數進行線性回歸以產生一斜率，可用於將測得的ΔOD/min轉換為FXa生成率：

$$\text{FXa生成率} = (\Delta OD/min) / (\text{斜率}_{\text{Fxa標準曲線}} * \text{反應時間})$$

【0389】 Michaelis-Menten穩態動力學參數是藉由將FXa生成率作為FX（受質）濃度的函數，以下列方程式進行擬合來測定：

$$\text{FXa生成率} = (k_{cat} * [FIXa]_t * [FX]_t) / (K_M + [FX]_t)$$

其中k_{cat}為酶的效率(min^{-1})，$[FIXa]_t$為總FIXa (酶)濃度(nM)，$[FX]_t$為總FX (受質)濃度(nM)，而K_M為Michaelis常數(nM)。

【0390】 表24列出了所測定之每個測試化合物的穩態動力學常數以及測試濃度。

表24：測得的穩態酶動力學常數

V_HH 多肽衍生物 ID / 抗體名稱	**測試的濃度 (nM)**	**K_M (nM)**	**k_{cat}/K_M (1/(min*nM))**
化合物#21	5	3.9	0.27
化合物#8	5	4.8	0.27
化合物#17	5	9.0	0.15
化合物#19	5	8.2	0.30
化合物#16	5	7.1	0.14

化合物#15	5	6.9	0.25
化合物#13	5	6.2	0.18
化合物#6	5	5.9	0.33
艾美賽珠單抗-SIA	300	53.5	0.03
游離 FIXa (10 nM)	N/A	281.5	0.00012

結論

【0391】 所測試的抗FIXa/FX雙特異性V_HH多肽衍生物在補充至最終濃度為5 nM時，都將K_M從游離FIXa的281.5 nM降低到3.9-9.0 nM之間，且進一步將催化效率從0.00012 $min^{-1}nM^{-1}$提高到0.14-0.33 $min^{-1}nM^{-1}$之間。相比之下，艾美賽珠單抗-SIA在300nM的時候，K_M下降到53.5 nM。

【0392】 降低的K_M值反映出FIX(a)/FX的組裝（空間排列）的改善，導致V_HH多肽衍生物的效價顯著增加。所有的化合物皆在預期的藥理學相關濃度下進行測試。

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【發明申請專利範圍】

【請求項1】一種促凝血V_HH多肽衍生物，包含：

一第一V_HH，其能夠與因子IX (SEQ ID NO:1)或其活化型結合，

一第二V_HH，其能夠與因子X (SEQ ID NO:2)結合，

一連接子(L_{1-2})，其連接所述第一V_HH及所述第二V_HH，及

一具有SEQ ID NO:9之C端延伸子(E)，

其具有下式（N端至C端）：

「第二V_HH」 – L_{1-2} – 「第一V_HH」 – E，

進一步包含與E接附之一第一延長部分及一第二延長部分，

其中

1)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:172)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:173)，且

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:174)；且

所述第二V_HH包含

CDR1：RYAMG (SEQ ID NO:152)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:153)，

CDR3：DDSVGDGYLDY (SEQ ID NO:154)；或

2)

所述第一V_HH包含

CDR1：IYTMS (SEQ ID NO:132)，

CDR2：GLRWTDSSTEYADSVKG (SEQ ID NO:133)，且

CDR3：DRSFLFAQALGATKNYEY (SEQ ID NO:134)；且

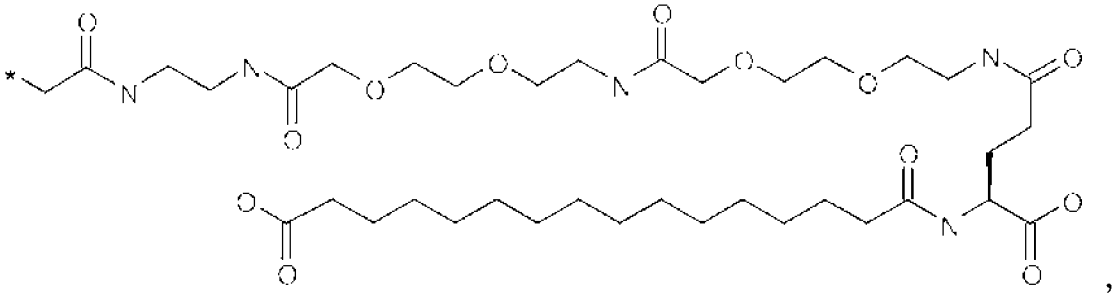
所述第二V_HH包含

CDR1：RLAMG (SEQ ID NO:128)，

CDR2：AISRRGGSTNYADSVKG (SEQ ID NO:129)，

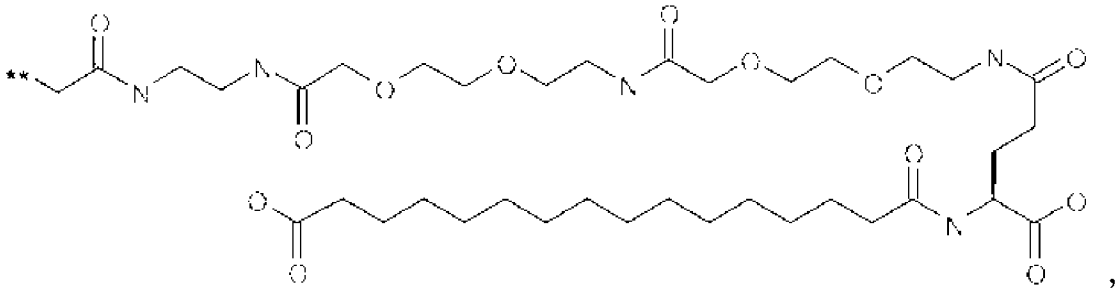
CDR3：DDSVGDGYLDY (SEQ ID NO:130)。

【請求項2】如請求項1所述之V_HH多肽衍生物，其包含SEQ ID NO:629且其中所述第一延長部分包含以下結構：



，

且其中所述第二延長部分包含以下結構：



，

其中「*」為SEQ ID NO:629之位置257的Cys (C)，且

其中「**」為SEQ ID NO:629之位置259的Cys (C)。

【請求項3】如請求項1所述之V_HH多肽衍生物，其中所述V_HH多肽衍生物係為雙特異性V_HH多肽衍生物。

【請求項4】一種醫藥組成物，其包含如請求項2所述之V_HH多肽衍生物，以及一醫藥學上可接受之賦形劑。

【請求項5】如請求項4所述之醫藥組成物，其中所述組成物包含N-(8-(2-羥基苯甲醯基)胺基)辛酸鹽。

【請求項6】如請求項5所述之醫藥組成物，其中所述N-(8-(2-羥基苯甲醯基)胺基)辛酸鹽為N-(8-(2-羥基苯甲醯基)胺基)辛酸鈉(SNAC)，且

其中所述組成物進一步包含菸鹼醯胺(NAM)。

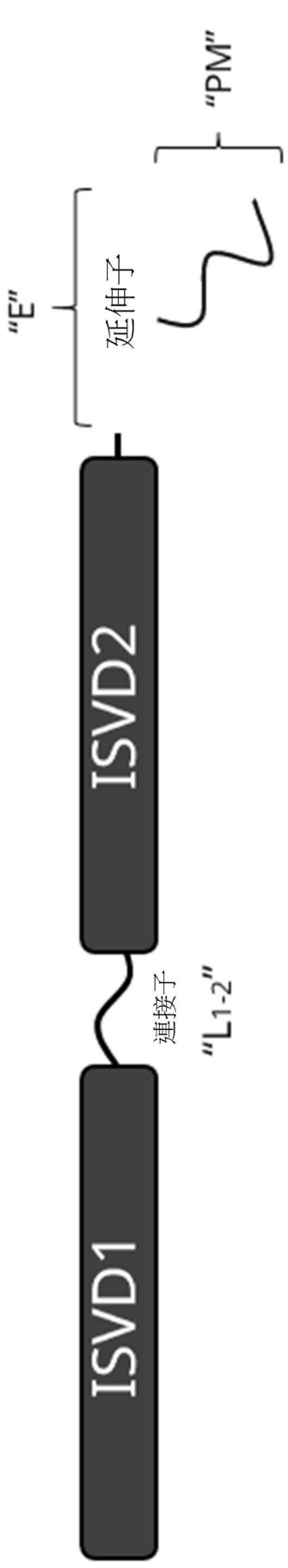
【請求項7】一種如請求項3所述之V_HH多肽衍生物的用途，其係用於製備用於治療具有或沒有抑制子之A型血友病或後天性A型血友病之藥物，其中該藥物係投予至有需要的患者。

【請求項8】一種如請求項4所述之醫藥組成物的用途，其係用於製備用於治療具有或沒有抑制子之A型血友病或後天性A型血友病之藥物，其中該藥物係投予至有需要的患者。

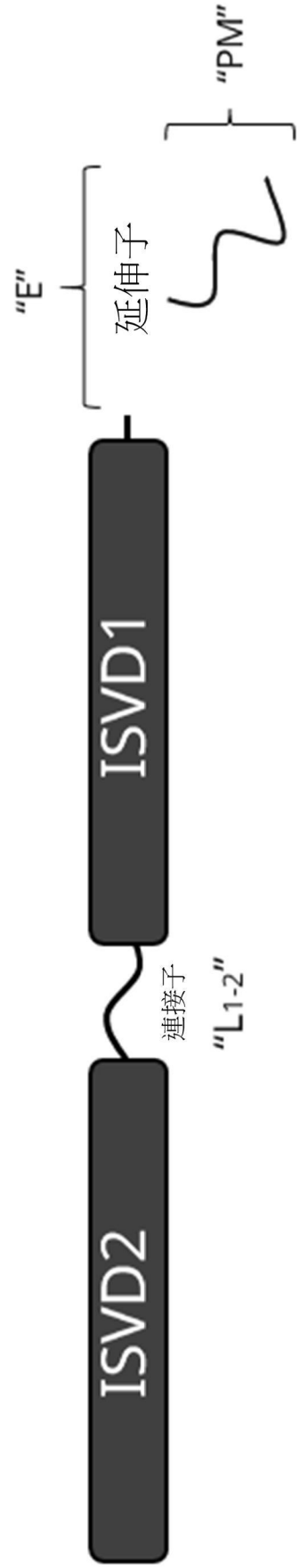
【請求項9】一種如請求項6所述之醫藥組成物的用途，其係用於製備用於治療具有或沒有抑制子之A型血友病或後天性A型血友病之藥物，其中該藥物係口服投予至有需要的患者。

【發明圖式】

【圖1a】



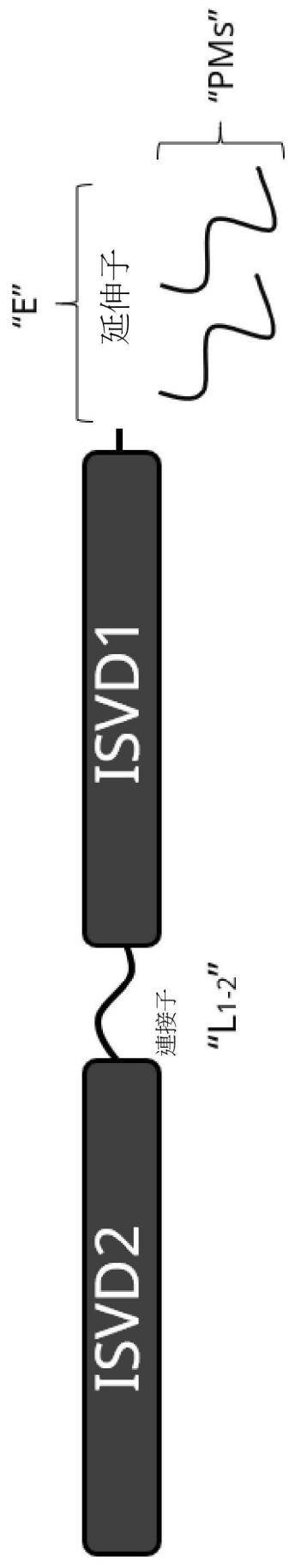
【圖 1b】



【圖 1c】



【圖 1d】



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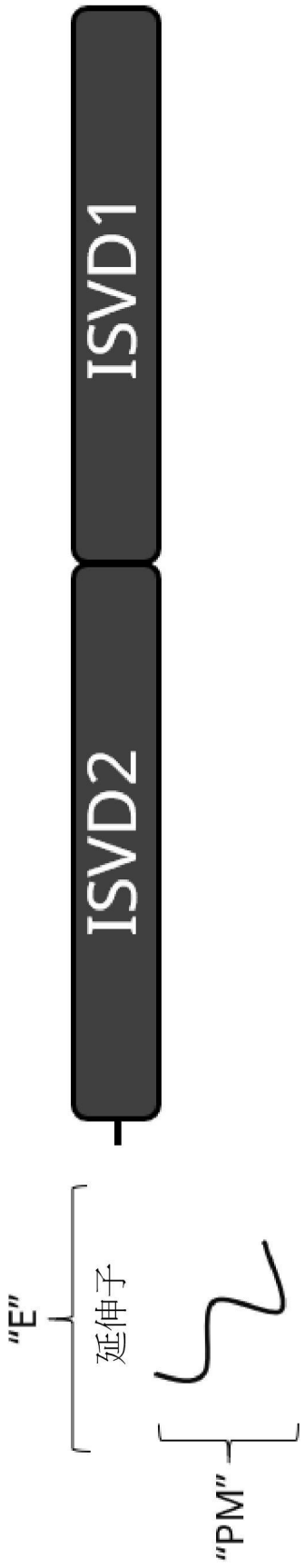

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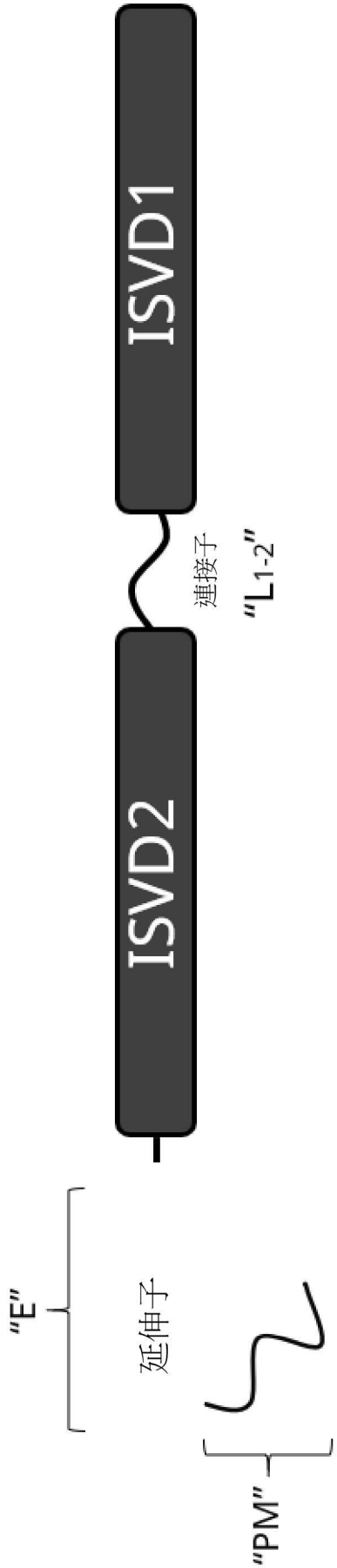

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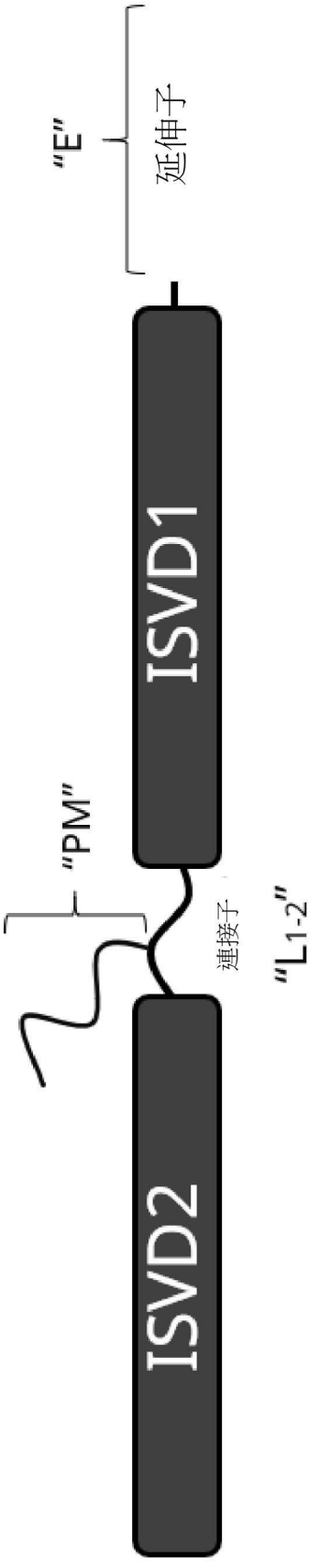

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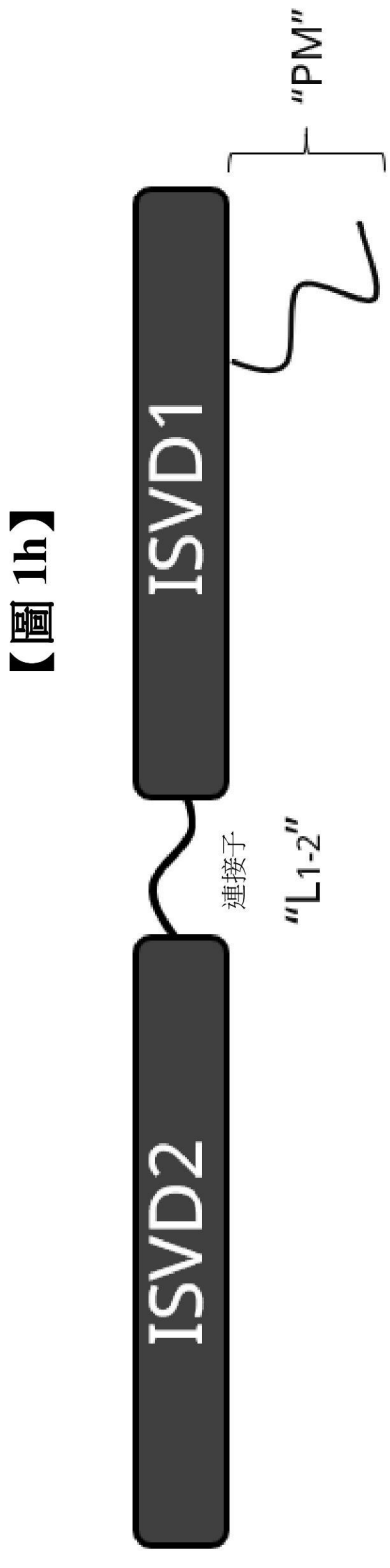

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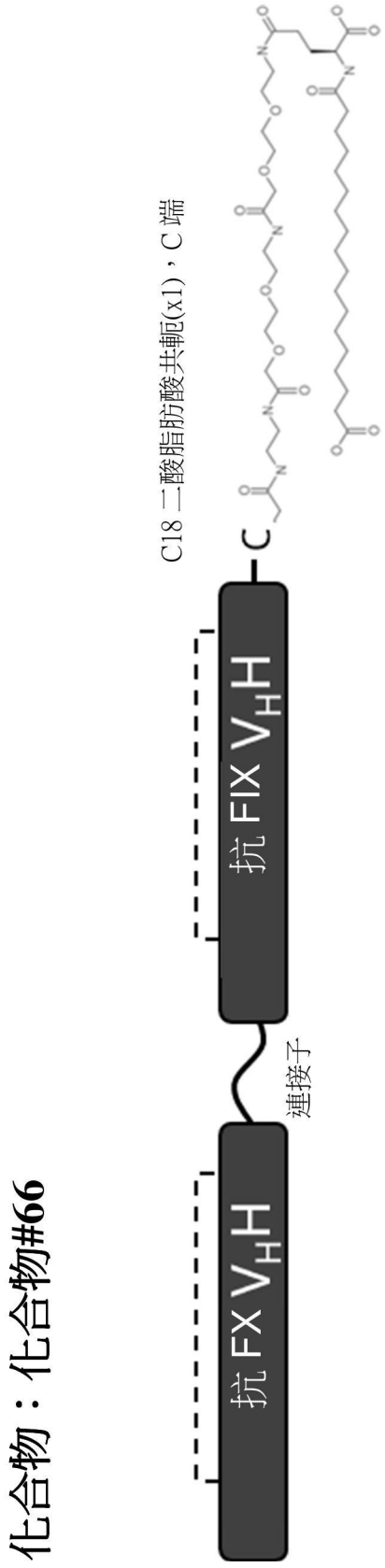

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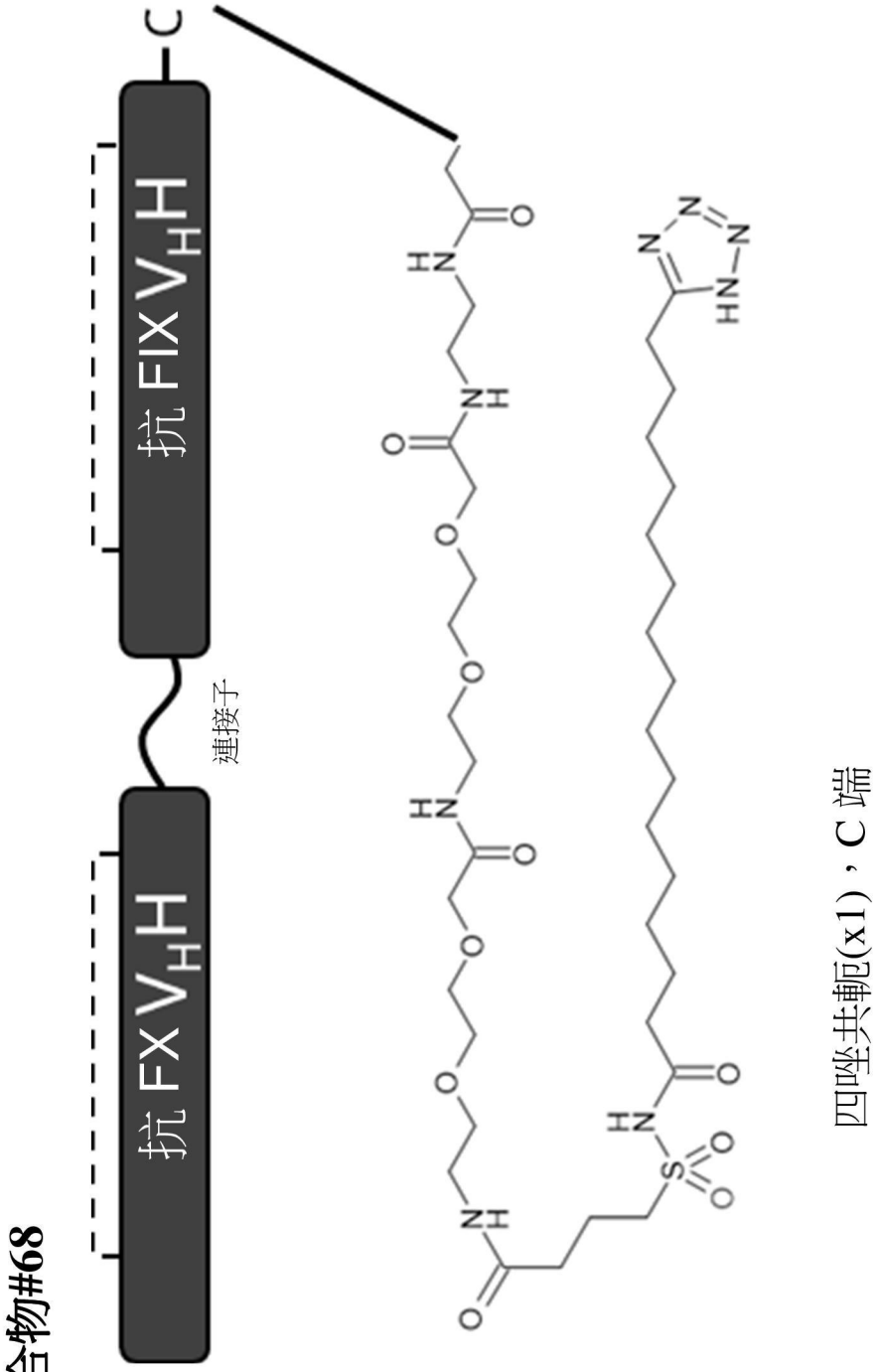

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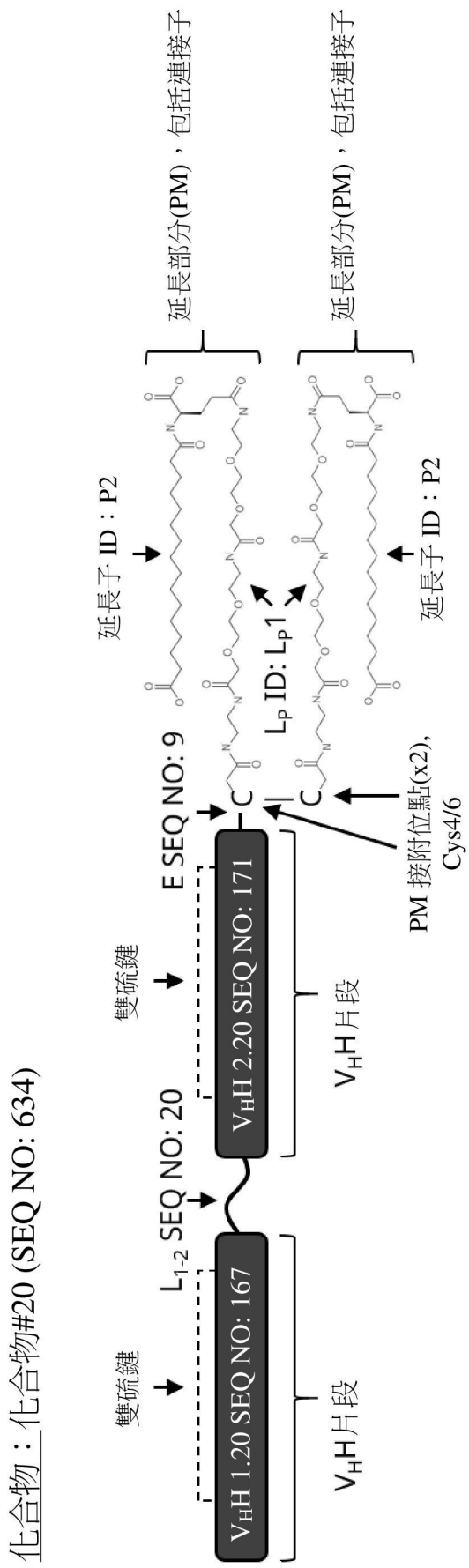
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E SEQ NO: 9
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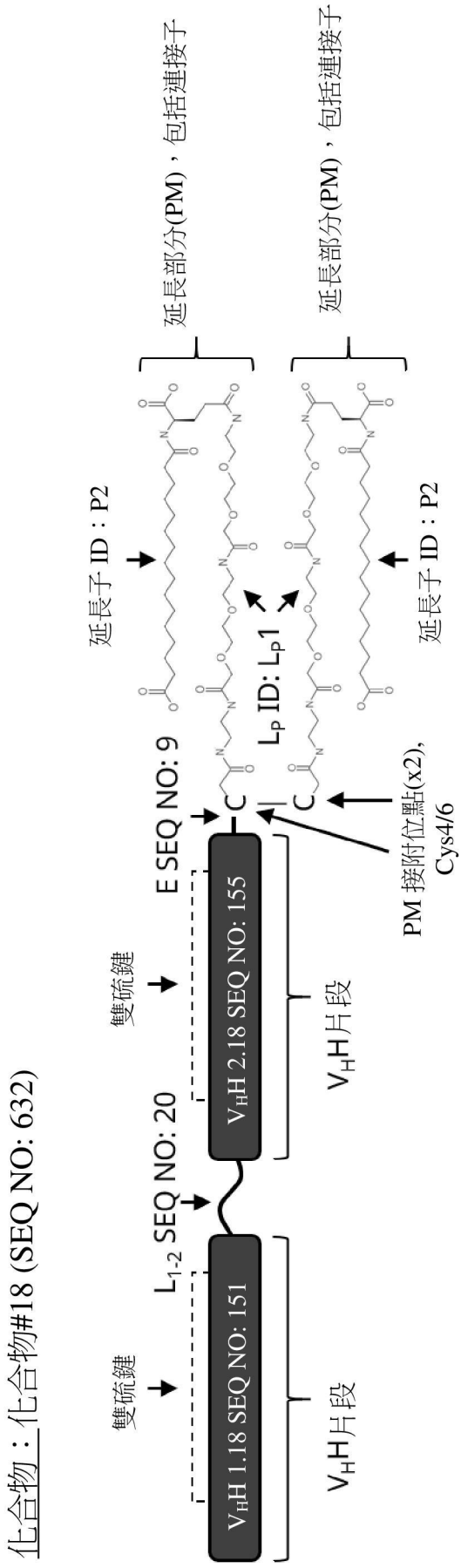
V_HH 2.20 SEQ NO: 171
V_HH片段
V_HH片段
L_P ID: L_P1
PM 接附位點(x2), Cys4/6
延長子 ID：P2
延長子 ID：P2
延長部分(PM)，包括連接子
延長部分(PM)，包括連接子

【圖3b】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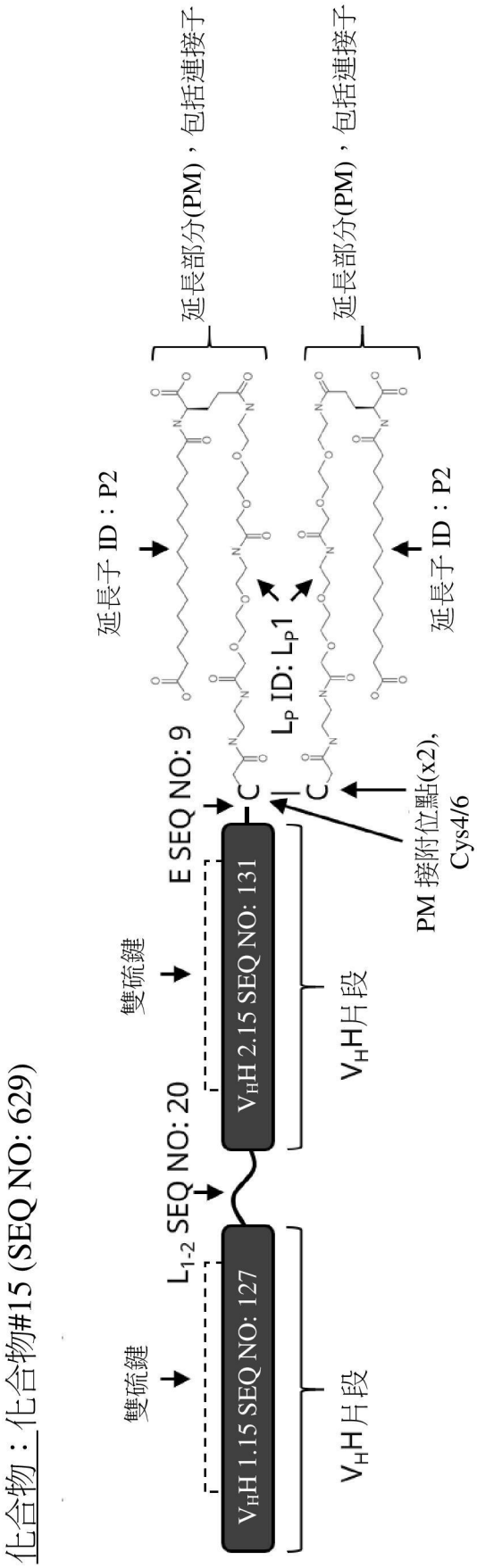
L_{1-2} SEQ NO: 20
雙硫鍵
E SEQ NO: 9
V_HH 2.18 SEQ NO: 155
V_HH片段
L_P ID: L_P1
PM 接附位點(x2), Cys4/6
延長子 ID：P2
延長部分(PM)，包括連接子
延長子 ID：P2
延長部分(PM)，包括連接子

【圖3c】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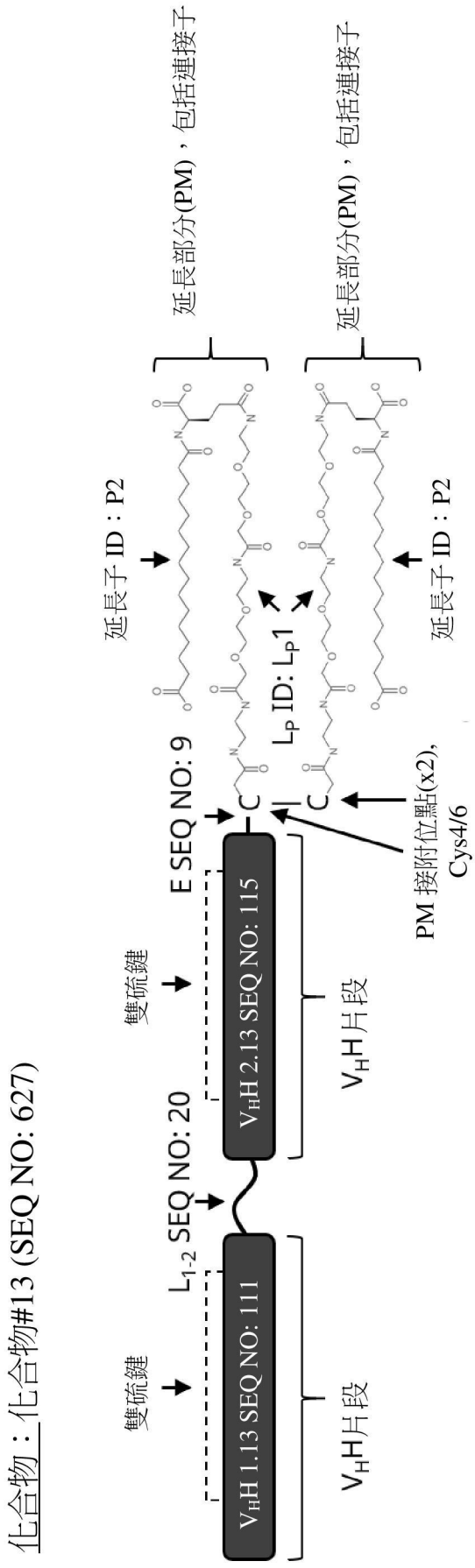
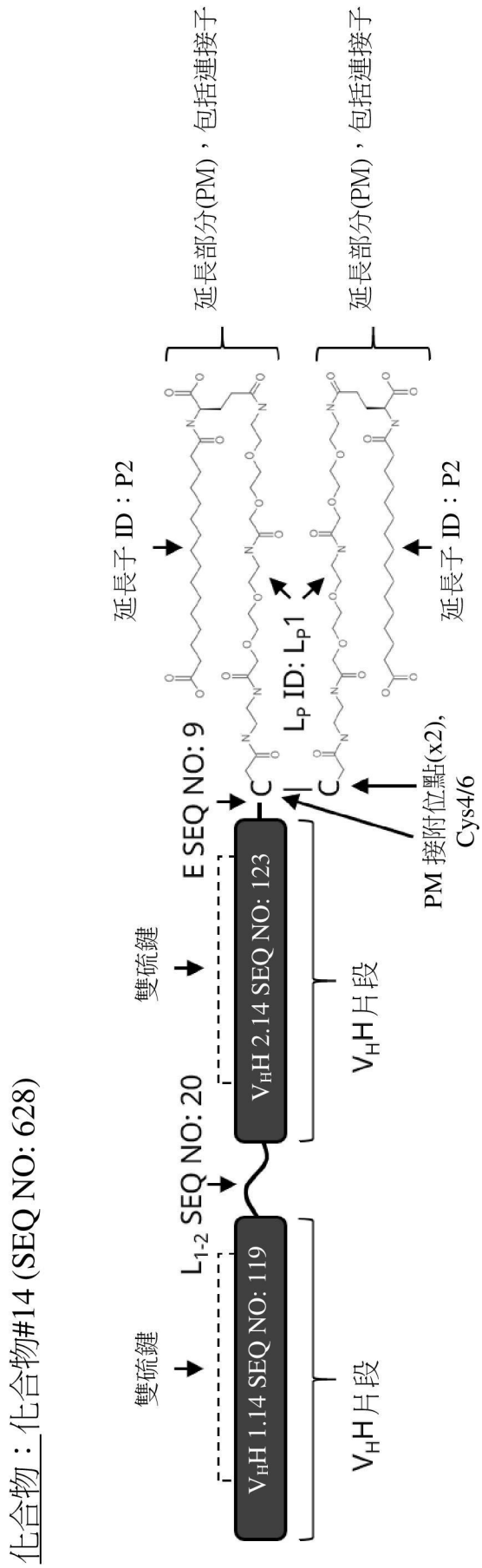
雙硫鍵
E SEQ NO: 9
VHH 2.15 SEQ NO: 131
VHH片段
LP ID: LP1
PM 接附位點(x2), Cys4/6
延長子 ID：P2
延長部分(PM)，包括連接子
延長子 ID：P2
延長部分(PM)，包括連接子

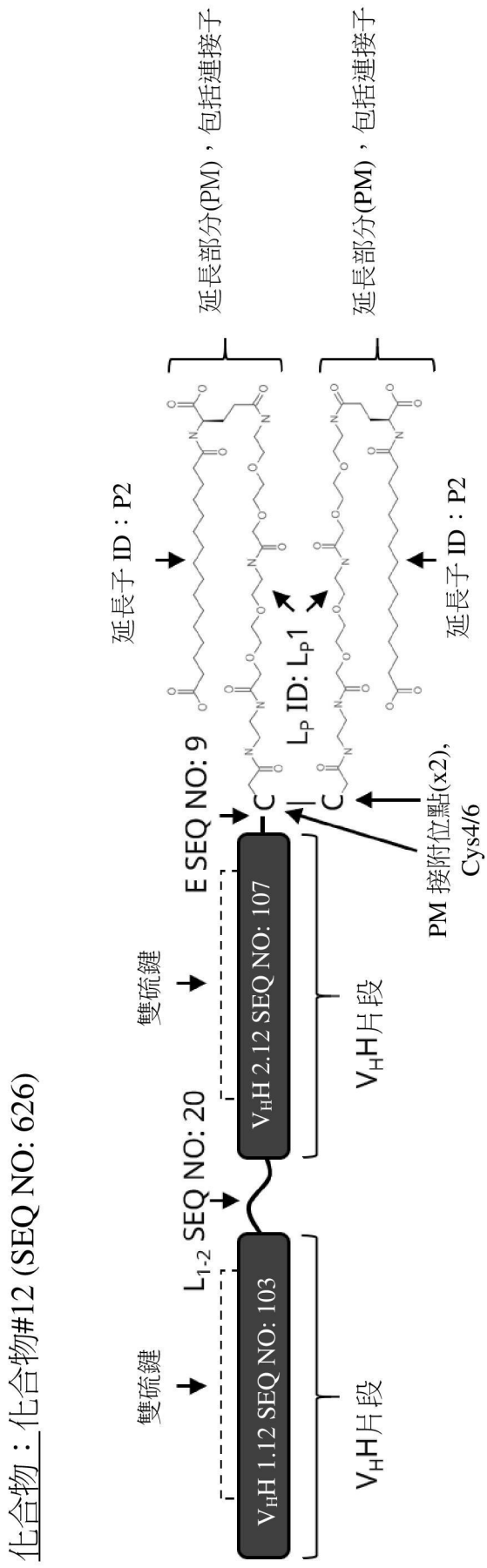
【圖3d】


化合物：化合物#13 (SEQ NO: 627)
雙硫鍵
V_HH片段
V_HH 1.13 SEQ NO: 111
L_{1-2} SEQ NO: 20
雙硫鍵
V_HH 2.13 SEQ NO: 115
V_HH片段
E SEQ NO: 9
L_P ID: L_P1
PM 接附位點(x2), Cys4/6
延長子 ID：P2
延長部分(PM)，包括連接子
延長子 ID：P2
延長部分(PM)，包括連接子

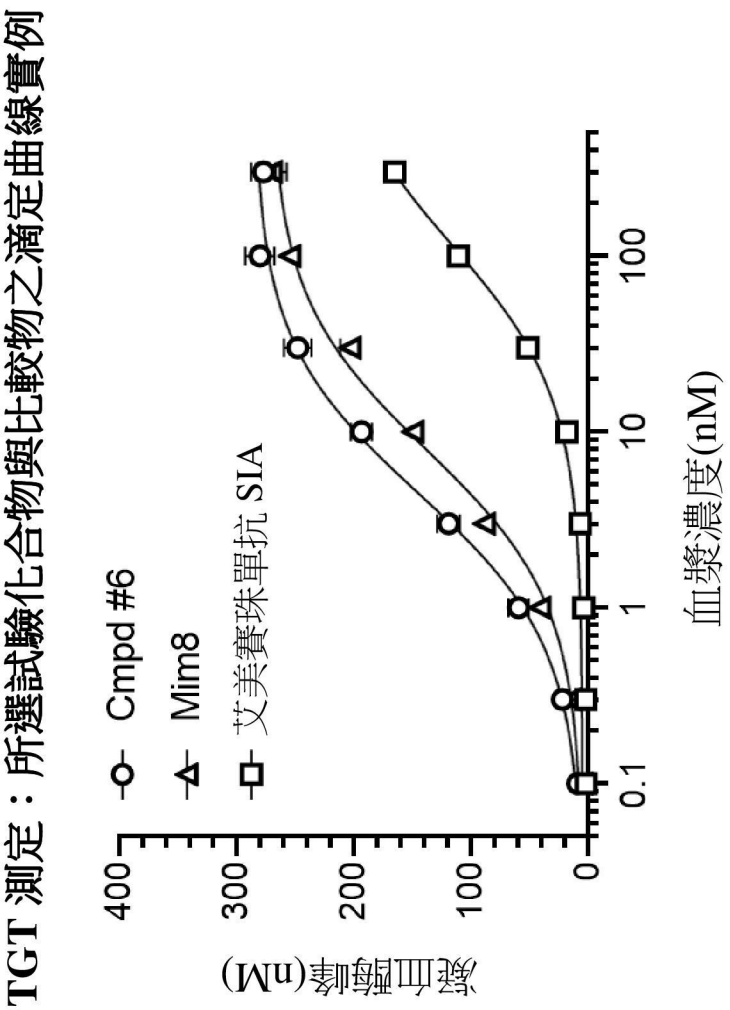
【圖3e】


化合物：化合物#14 (SEQ NO: 628)
雙硫鍵
L1-2 SEQ NO: 20
VHH 1.14 SEQ NO: 119
VHH片段
雙硫鍵
E SEQ NO: 9
VHH 2.14 SEQ NO: 123
VHH片段
LP ID: LP1
PM 接附位點(x2), Cys4/6
延長子 ID：P2
延長部分(PM)，包括連接子
延長子 ID：P2
延長部分(PM)，包括連接子

【圖3f】


化合物：化合物#12 (SEQ NO: 626)
雙硫鍵
L_{1-2} SEQ NO: 20
E SEQ NO: 9
雙硫鍵
V_HH 1.12 SEQ NO: 103
V_HH 2.12 SEQ NO: 107
V_HH片段
V_HH片段
L_P ID: L_P1
PM 接附位點(x2), Cys4/6
延長子 ID：P2
延長子 ID：P2
延長部分(PM)，包括連接子
延長部分(PM)，包括連接子

【圖 4】


TGT 測定：所選試驗化合物與比較物之滴定曲線實例
Cmpd #6
Mim8
艾美賽珠單抗 SIA
凝血酶峰(nM)
400
300
200
100
0
0.1
1
10
100
血漿濃度(nM)

【圖5a】

結合FIX(a)之ISVD序列的序列比對，CDR序列係以黑體及下劃線顯示

Cmpd20_aFIX	EVQLVESGGGVVQPGGSLRLSCAASGRTFN**IYTMS**WFRQAPGQEREFVA**GLRWTDSSTEYADSVKG**RFTISRDNSKNTVYLQMNSLRPEDTALYYCAA**DRSFLFAQALGATKNYEY**WGQGTLVTVSS
Cmpd18_aFIX	EVQLVESGGGVVQPGGSLRLSCAASGRTFN**IYTMS**WFRQDPGQEREFVA**GLRWTDSSTEYADSVKG**RFTISRDNSKNTVYLQMNSLRPEDTALYYCAA**DRSFLFAQALGATKNYEY**WGQGTLVTVSS
Cmpd15_aFIX	EVQLVESGGGVVQPGGSLQLSCAASGRTFN**IYTMS**WFRQAPGQEREFVA**GLRWTDSSTEYADSVKG**RFTISRDNSKNTVYLQMDSLRPEDTALYYCAA**DRSFLFAQALGATKNYEY**WGQGTLVTVSS
Cmpd13_aFIX	EVQLVESGGGVVQPGGSLQLSCAASGRTFN**IYTMS**WFRQDPGQEREFVA**GLRWTDSSTEYADSVKG**RFTISRDNSKNTVYLQMDSLRPEDTALYYCAA**DRSFLFAQALGATKNYEY**WGQGTLVTVSS
Cmpd14_aFIX	EVQLVESGGGVVQPGGSLQLSCAASGRTFN**IYTMS**WFRQAPGQEREFVA**GLRWTDSSTEYADSVKG**RFTISRDNSKNTVYLQMDSLRPEDTALYYCAA**DRSFLFAQALGATKNYEY**WGQGTLVTVSS
Cmpd12_aFIX	EVQLVESGGGVVQPGGSLQLSCAASGRTFN**IYTMS**WFRQDPGQEREFVA**GLRWTDSSTEYADSVKG**RFTISRDNSKNTVYLQMDSLRPEDTALYYCAA**DRSFLFAQALGATKNYEY**WGQGTLVTVSS

【圖5b】

結合FX之ISVD序列的序列比對，CDR序列係以黑體及下劃線顯示

Cmpd20_aFX	DVQLVESGGGVVQPGGSLQLSCAASGGTKS**RYAMG**WFRQAPGQEREWVA**AISRRGGSTNYADSVKG**RFTISRDNSKNTVYLQMDSLRPEDTALYYCAA**DDSVGDGYLDY**WGQGTLVTVSS
Cmpd18_aFX	DVQLVESGGGVVQPGGSLQLSCAASGGTKS**RYAMG**WFRQAPGQEREWVA**AISRRGGSTNYADSVKG**RFTISRDNSKNTVYLDMDDLRPEDTALYYCAA**DDSVGDGYLDY**WGQGTLVTVSS
Cmpd15_aFX	DVQLVESGGGVVQPGGSLQLSCAASGGTKR**RLAMG**WFRQAPGQEREWVA**AISRRGGSTNYADSVKG**RFTISRDNSKNTVYLQMDSLRPEDTALYYCAA**DDSVGDGYLDY**WGQGTLVTVSS
Cmpd13_aFX	DVQLVESGGGVVQPGGSLQLSCAASGGTKR**RLAMG**WFRQAPGQEREWVA**AISRRGGSTNYADSVKG**RFTISRDNSKNTVYLDMDDLRPEDTALYYCAA**DDSVGDGYLDY**WGQGTLVTVSS
Cmpd14_aFX	DVQLVESGGGVVQPGGSLQLSCAASGGTKR**RLAMG**WFRQAPGQEREFVA**AISRRGGSTNYADSVKG**RFTISRDNSKNTVYLQMDSLRPEDTALYYCAA**DDSVGDGYLDY**WGQGTLVTVSS
Cmpd12_aFX	DVQLVESGGGVVQPGGSLQLSCAASGGTKR**RLAMG**WFRQAPGQEREFVA**AISRRGGSTNYADSVKG**RFTISRDNSKNTVYLDMDDLRPEDTALYYCAA**DDSVGDGYLDY**WGQGTLVTVSS