Novel JNK inhibitor molecules

The present invention relates to the field of enzyme inhibition, in particular to (poly-)peptide inhibitors of c-Jun amino terminal kinase (JNK). The present invention furthermore relates to methods for raising antibodies against such (poly-)peptide inhibitors as well as to the respective antibodies and cells producing the same.

The c-Jun amino terminal kinase (JNK) is a member of the stress-activated group of mitogen-activated protein (MAP) kinases. These kinases have been implicated in the control of cell growth and differentiation, and, more generally, in the response of cells to environmental stimuli. The JNK signal transduction pathway is activated in response to environmental stress and by the engagement of several classes of cell surface receptors. These receptors can include cytokine receptors, serpentine receptors and receptor tyrosine kinases. In mammalian cells, JNK has been implicated in biological processes such as oncogenic transformation and mediating adaptive responses to environmental stress. JNK has also been associated with modulating immune responses, including maturation and differentiation of immune cells, as well as effecting programmed cell death in cells identified for destruction by the immune system. The mitogen-activated protein kinase (MAPK) p38alpha was shown to negatively regulate the cell proliferation by antagonizing the JNK-c-Jun-pathway. The mitogen-activated protein kinase (MAPK) p38alpha therefore appears to be active in suppression of normal and cancer cell proliferation (see e.g. Hui *et al.*, Nature Genetics, Vol 39, No. 6, June 2007). It was also shown, that c-Jun N-terminal Kinase (JNK) is involved in neuropathic pain produced by spinal nerve ligation (SNL), wherein SNL induced a slow and persistent activation of JNK, in particular JNK1, whereas p38 mitogen-activated protein kinase activation was found in spinal microglia after SNL, which had fallen to near basal level by 21 days (Zhuang et al., The Journal of Neuroscience, March 29, 2006, 26(13):3551-3560)).

Inhibitors of the JNK signaling pathway as already known in the prior art, particularly include e.g. upstream kinase inhibitors (for example, CEP-1347), small chemical inhibitors of JNK (SP600125

and AS601245), which directly affect kinase activity e.g. by competing with the ATP-binding site of the protein kinase, and peptide inhibitors of the interaction between JNK and its substrates (see e.g. Kuan et al., Current Drug Targets – CNS & Neurological Disorders, February 2005, vol. 4, no. 1, pp. 63-67; WO 2007/031280; all incorporated herewith by reference). WO 2007/031280 discloses small cell permeable fusion peptides, comprising a so-called TAT transporter sequence derived from the basic trafficking sequence of the HIV-TAT protein and an amino acid inhibitory sequence of IB1.

WO 2007/031280 discloses in particular two specific sequences, L-TAT-IB1 (GRKKRRQRRRPPRPKRPTTLNLFPQVPRSQD, herein SEQ ID NO: 196) and D-TAT-IB1 (dqsrpvqpflnlttprkprpprrrqrrkkrg; herein SEQ ID NO: 197), the latter being the retro-inverso sequence of L-TAT-IB1. Due to the HIV TAT derived transporter sequence, these fusion peptides are more efficiently transported into the target cells, where they remain effective until proteolytic degradation.

Since ATP independent peptide inhibitors of JNK are usually more specific inhibitors, they are frequently the first choice if it comes to inhibiting JNK. However, even the peptide inhibitors disclosed in WO 2007/031280 are not optimal. For example, compound L-TAT-IB1 (herein SEQ ID NO: 196) which consists of L amino acids only, is quickly proteolytically degraded. In order to overcome this problem the inventors of WO 2007/031280 also suggested D-TAT-IB1 (herein SEQ ID NO: 197), which comprises D amino acids. To be more precise, D-TAT-IB1 exhibits the retro-inverso sequence of L-TAT-IB1. Incorporation of D-amino acids is made difficult by the fact that the change in stereochemistry may lead to a loss of function. The retro-inverso approach may be employed to reduce said risk because the use of i) only D-amino acids ii) but in the inverse peptide sequence may more likely yield an acceptable conformational analogue to the original peptide than incorporating one or more D-amino acids into the original sequence. In the case of WO 2007/031280 this approach resulted nevertheless in a significant decrease in inhibitory capacity in comparison to L-TAT-IB1 (see Fig. 4). Additionally, the retro-inverso peptide is extremely stable towards proteolytic digestion with the consequence that controlled digestions, for example in time sensitive experiments, are hardly possible.

Therefore, there is still a need in the art for peptide inhibitors of JNK which are more stable than for example L-TAT-IB1 (herein SEQ ID NO: 196). On the other hand there is a need for peptide inhibitors of JNK which are more active while less stable than for example D-TAT-IB1 (herein SEQ ID NO: 197).

Thus, the problem to be solved by the present invention was to provide further (peptide) inhibitors of JNK which are preferably less sensitive to proteolytic degradation than L-TAT-IB1 as disclosed in WO 2007/031280, but are preferably at the same time more sensitive to proteolytic degradation and/or more active than D-TAT-IB1 as disclosed in WO 2007/031280.

The object of the present invention is solved by the inventor by means of the subject-matter set out in the appended claims.

In the following a brief description of the appended figures will be given. The figures are intended to illustrate the present invention in more detail. However, they are not intended to limit the subject matter of the invention in any way.

Fig. 1: Illustration of the inhibitory efficacy of several JNK inhibitors according to the present invention, which was investigated by in vitro AlphaScreen assay (Amplified Luminescence Proximity Homogeneous-Screen Assay).
Fig.1A: Inhibition of JNK1 by SEQ ID NOs: 193, 2, 3, 5, 6, and 7.
Fig.1B: Inhibition of JNK2 by SEQ ID NOs: 193, 2, 3, 5, 6, and 7.
Fig.1C: Inhibition of JNK3 by SEQ ID NOs: 193, 2, 3, 5, 6, and 7.

Fig. 2: Table illustrating the inhibitory efficacy of several JNK inhibitors (SEQ ID NOs: 193, 2, 3, 5, 6, and 7) according to the present invention. Given are the IC50 values in the nM range, the respective standard error of the mean and the number of experiments performed (n).

Fig. 3: Illustration of the inhibitory efficacy of several JNK inhibitors according to the present invention, which are fusion proteins of a JNK inhibitory (poly-)peptide sequence and a transporter sequence. The inhibitory efficacy was determined by means of in vitro AlphaScreen assay (Amplified Luminescence Proximity Homogeneous-Screen Assay).
Fig.3A: Inhibition of JNK1 by SEQ ID NOs: 194, 195, 172, 200, 46, 173, 174, 175, 176, 177, 178, 179, 180, 181 and 197.
Fig.3B: Inhibition of JNK2 by SEQ ID NOs: 194, 195, 172, 200, 46, 173, 174, 175, 176, 177, 178, 179, 180, 181 and 197.
Fig.3C: Inhibition of JNK3 by SEQ ID NOs: 194, 195, 172, 200, 46, 173, 174, 175, 176, 177, 178, 179, 180, 181 and 197.
Fig.3D: Inhibition of JNK1 by SEQ ID NOs: 194, 195, 172, 200, 46, 182, 183, 184, 185, 186, 187, 188, 189, 190 and 197.

Fig.3E: Inhibition of JNK2 by SEQ ID NOs: 194, 195, 172, 200, 46, 182, 183, 184, 185, 186, 187, 188, 189, 190 and 197.

Fig.3F: Inhibition of JNK3 by SEQ ID NOs: 194, 195, 172, 200, 46, 182, 183, 184, 185, 186, 187, 188, 189, 190 and 197.

Fig. 4: Table illustrating the inhibitory efficacy of several JNK inhibitors according to the present invention, which are fusion proteins of a JNK inhibitory (poly-)peptide sequence and a transporter sequence. Given are the IC50 values in the nM range, the respective standard error of the mean (SEM) and the number of experiments performed (n).

Fig. 5: Stability of JNK inhibitors with SEQ ID NOs: 172, 196 and 197 in 50% human serum. The JNK inhibitor with SEQ ID NO: 196 was totally degraded into amino acids residues within 6 hours (A). The JNK inhibitor with SEQ ID NO: 172 was completely degraded only after 14 days (B). The JNK inhibitor with SEQ ID NO: 197 was stable at least up to 30 days (B).

Fig. 6: shows internalizations experiments using TAT derived transporter constructs with D-amino acid/L-amino acid pattern as denoted in SEQ ID NO: 30. The transporter sequences analyzed correspond to SEQ ID NOs: 52-94 plus SEQ ID NOs: 45, 47, 46, 43 and 99 (Fig 6a) and SEQ ID NOs: 100-147 (Fig. 6b). As can be seen, all transporters with the consensus sequence rXXXrXXXr (SEQ ID NO: 31) showed a higher internalization capability than the L-TAT transporter (SEQ ID NO: 43). Hela cells were incubated 24 hours in 96well plate with 10mM of the respective transporters. The cells were then washed twice with an acidic buffer (0.2M Glycin, 0.15M NaCl, pH 3.0) and twice with PBS. Cells were broken by the addition of RIPA lysis buffer. The relative amount of internalized peptide was then determined by reading the fluorescence intensity (Fusion Alpha plate reader; PerkinElmer) of each extract followed by background subtraction.

Fig. 7 The JNK inhibitor with the sequence of SEQ ID NO: 172 blocks LPS-induced cytokine and chemokine release in THP1- PMA-differentiated macrophages. Fig. 7A: TNF release (THP1pma 6h 3ng/ml LPS); Fig. 7B: TNFa release (THP1pma 6h 10ng/ml LPS); Fig. 7C: IL 6 release (THP1pma 6h 10ng/ml LPS); Fig. 7D: MCP1 release (THP1pma 6h 3ng/ml LPS).

Fig. 8 The JNK inhibitor of SEQ ID NO: 172 blocks LPS-induced IL6 release in THP1 differentiated macrophages with higher potency than D-TAT-IB1 (SEQ ID NO: 197), dTAT (SEQ ID NO: 45) and SP 600125. LPS was added for 6h (10 ng/ml).

Fig. 9 The JNK inhibitor of SEQ ID NO: 172 blocks LPS-induced TNFα release in THP1 differentiated macrophages with higher potency than D-TAT-IB1 (SEQ ID NO: 197), dTAT (SEQ ID NO: 45) and SP 600125. LPS was added for 6h (10 ng/ml).

Fig. 10 The JNK inhibitor of SEQ ID NO: 172 blocks LPS-induced IL-6 release in PMA differentiated macrophages with higher potency than D-TAT-IB1 (SEQ ID NO: 197) and L-TAT-IB1 (SEQ ID NO: 196). LPS was added for 6h.

Fig. 11 The JNK inhibitor of SEQ ID NO: 172 blocks LPS-induced TNFα release in PMA differentiated macrophages with higher potency than D-TAT-IB1 (SEQ ID NO: 197) and L-TAT-IB1 (SEQ ID NO: 196).

Fig. 12 The JNK inhibitor of SEQ ID NO: 172 blocks LPS-induced TNFα release in Primary Rat Whole Blood Cells at 3 ng/ml. Given are the results for the control, 1 μM of SEQ ID NO: 172, 3 μM of SEQ ID NO: 172, and 10 μM of SEQ ID NO: 172 at different levels of LPS (ng/ml).

Fig. 13 The JNK inhibitor of SEQ ID NO: 172 blocks IL2 secretion by primary human T-cells in response to PMA/Ionomycin.

Fig. 14 The JNK inhibitor of SEQ ID NO: 172 blocks IL2 secretion by primary human T-cells in response to CD3/CD28 stimulation. The JNK inhibitors used are Indicated by their SEQ ID NO: 172 and 197.

Fig. 15 Dose-dependent inhibition by JNK inhibitor with SEQ ID NO: 172 of CD3/CD28-induced IL-2 release in primary rat lymph-nodes purified T cells. Control rat were sacrificed and lymph-nodes were harvested. T cells further were purified (using magnetic negative selection) and plated into 96-well plates at 200.000 cells/well. Cells were treated with anti-rat CD3 and anti-rat CD28 antibodies (2μg/mL). JNK inhibitor with SEQ ID NO: 172 was added to the cultures 1h before CD3/CD28 treatment and IL-2 release was assessed in supernatant 24h after treatment.

Fig. 16 Dose-dependent inhibition of CD3/CD28-induced IL-2 release in primary rat lymph-nodes purified T cells: Comparison of several JNK inhibitors, namely SEQ ID NOs: 172, 197 and SP600125.

Fig. 17 Dose dependent inhibition of IL-2 release in rat whole blood stimulated with PMA + ionomycin. JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10 µM 1h before stimulation with PMA + ionomycin. Three doses of activators were added (25/500 ng/mL, 50/750 ng/mL and 50/1000 ng/mL) for 4h. IL-2 release was assessed in supernatant. JNK inhibitor with SEQ ID NO: 172 at 10µM did efficiently reduce PMA-iono-induced IL-2 release at the three tested activator concentrations.

Fig. 18 JNK inhibition and IL-6 release in human whole blood. The JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10µM 1h before whole blood stimulation with LPS (0.02ng/mL) for 4 hours. The JNK inhibitor with SEQ ID NO: 172 did reduce the LPS-induced IL-6 release in a dose-dependent manner.

Fig. 19 JNK inhibition and IL-2 release in human whole blood. The JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10µM 1h before whole blood stimulation with PMA+ionomycin (25/700ng/mL, 50/800ng/ml and 50/1000ng/mL) for 4 hours. The JNK inhibitor with SEQ ID NO: 172 did reduce the PMA+ionomycin -induced IL-2 release in a dose-dependent manner.

Fig. 20 JNK inhibition and IFN-γ release in human whole blood. The JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10µM 1h before whole blood stimulation with PMA+ionomycin (25/700ng/mL, 50/800ng/ml and 50/1000ng/mL) for 4 hours. The JNK inhibitor with SEQ ID NO: 172 did reduce the PMA+ionomycin -induced IFN-γ release in a dose-dependent manner.

Fig. 21 JNK inhibition and TNF-α release in human whole blood. The JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10µM 1h before whole blood stimulation with PMA+ionomycin (25/700ng/mL, 50/800ng/ml and 50/1000ng/mL) for 4 hours. The JNK inhibitor with SEQ ID NO: 172 did reduce the PMA+ionomycin -induced TNF-α release in a dose-dependent manner.

Fig. 22 JNK inhibition and TNF-α release in human whole blood. The JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10µM 1h before whole blood stimulation with PHA-L (5µg/mL) for 3 days. The JNK inhibitor with SEQ ID NO: 172 did reduce the PHA-L-induced TNF-α release in a dose-dependent manner.

Fig. 23 JNK inhibition and IL-2 release in human whole blood. The JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10μM 1h before whole blood stimulation with PHA-L (5μg/mL) for 3 days. The JNK inhibitor with SEQ ID NO: 172 did reduce the PHA-L-induced IL-2 release in a dose-dependent manner.

Fig. 24 JNK inhibition and TNF-α release in human whole blood. The JNK inhibitor with SEQ ID NO: 172 was added at three different concentrations, namely 1, 3 and 10μM 1h before whole blood stimulation with CD3 +/- CD28 antibodies (2μg/mL) for 3 days. The JNK inhibitor with SEQ ID NO: 172 did reduce the CD3/CD28-induced TNF-α release in a dose-dependent manner.

JNK inhibitors

In a first aspect the present invention relates to a JNK inhibitor, which comprises an inhibitory (poly-)peptide sequence according to the following general formula:

X1-X2-X3-R-X4-X5-X6-L-X7-L-X8 (SEQ ID NO: 1),

wherein X1 is an amino acid selected from amino acids R, P, Q and r,
wherein X2 is an amino acid selected from amino acids R, P, G and r,
wherein X3 is an amino acid selected from amino acids K, R, k and r,
wherein X4 is an amino acid selected from amino acids P and K,
wherein X5 is an amino acid selected from amino acids T, a, s, q, k or is absent,
wherein X6 is an amino acid selected from amino acids T, D and A,
wherein X7 is an amino acid selected from amino acids N, n, r and K; and
wherein X8 is an amino acid selected from F, f and w,

with the proviso that at least one, at least two, at least three, at least four, at least five or six of the amino acids selected from the group consisting of X1, X2, X3, X5, X7 and X8 is/are a D-amino acid(s), preferably with the proviso that at least one, at least two, at least three or four of the amino acids selected from the group consisting of X3, X5, X7 and X8 is/are a D-amino acid(s).

The inhibitory (poly-)peptide sequence of the JNK inhibitor according to the present invention comprises L-amino acids and in most embodiments D-amino acids. Unless specified otherwise, L-amino acid residues are indicated herein in capital letters, while D amino acid residues are indicated in small letters. Glycine may be indicated in capital or small letters (since there is no D-

or L-glycine). The amino acid sequences disclosed herein are always given from N- to C-terminus (left to right) unless specified otherwise. The given amino acid sequence may be modified or unmodified at the C- and/or N-terminus, e.g. acetylation at the C-terminus and/or amidation or modification with cysteamide at the N-terminus. For sake of clarity such possible but entirely optional modifications at the C- and/or N-terminus of the amino acid sequences disclosed herein are for sake of clarity not specifically indicated.

The JNK inhibitors of the present invention are (poly-)peptide inhibitors of the c-Jun N-terminal kinase (JNK). Said inhibitors inhibit the kinase activity of c-Jun N-terminal kinase (JNK), i.e. prevent or reduce the extent of phosphorylation of JNK substrates such as c-Jun, ATF2 and/or Elk-1. A person skilled in the art will understand that the term "inhibitor", as used herein, does not comprise compounds which irreversibly destroy the c-Jun N-terminal kinase (JNK) molecule and/or kinase activity. Furthermore, the term "inhibiting JNK activity" as used herein, refers to the inhibition of the kinase activity of c-Jun N-terminal kinase (JNK).

Furthermore, as used herein, a JNK inhibitor comprises at least one functional unit of a polymer of amino acids, i.e. a (poly-)peptide sequence. Moreover, this at least one functional polymer of amino acids provides for inhibition of JNK activity. The amino acid monomers of said inhibitory (poly-)peptide sequence are usually linked to each other via peptide bonds, but (chemical) modifications of said peptide bond(s) or of side chain residues may be tolerable, provided the inhibitory activity (inhibition of JNK activity) is not totally lost, i.e. the resulting chemical entity still qualifies as JNK inhibitor as functionally defined herein. The term "(poly-)peptide" shall not be construed as limiting the length of the (poly-)peptide unit. Preferably, the inhibitory (poly-)peptide sequence of the JNK inhibitors of the present invention is less than 500, 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380, 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, 270, 260, 250, 240, 230, 220, 210, 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, or less than 12 amino acids long. Preferably, the inhibitory (poly-)peptide sequence does not have less than 10 amino acid residues, more preferably not less than 11 amino acid residues.

Furthermore, a "JNK inhibitor" of the present invention inhibits JNK activity, e.g. exhibits with regard to the inhibition of human JNK mediated phosphorylation of a c-Jun substrate (SEQ ID NO: 198) an IC 50 value of:

a) less than 3000 nM, more preferably less than 2000 nM, even more preferably less than 1000 nM, even more preferably less than 500 nM, even more preferably less than 250 nM, even more preferably less than 200 nM, even more preferably less than 150 nM, most preferably less than 100 nM with regard to inhibition of human JNK1,

b) less than 3000 nM, more preferably less than 2000 nM, even more preferably less than 1000 nM, even more preferably less than 500 nM, even more preferably less than 250 nM, even more preferably less than 200 nM, even more preferably less than 150 nM, most preferably less than 100 nM with regard to inhibition of human JNK2, and/or

c) less than 3000 nM, more preferably less than 2000 nM, even more preferably less than 1000 nM, even more preferably less than 500 nM, even more preferably less than 250 nM, even more preferably less than 200 nM, even more preferably less than 150 nM, most preferably less than 100 nM with regard to inhibition of human JNK3.

For some applications it is preferred that the inhibitor inhibits human JNK2 and/or human JNK3 according to the above definition, but not JNK1 according to the above definition.

Whether JNK activity is inhibited or not, may easily be assessed by a person skilled in the art. There are several methods know in the art. One example is a radioactive kinase assay or a non-radioactive kinase assay (e.g. Alpha screen test; see for example Guenat et al. J Biomol Screen, 2006; 11: pages 1015-1026).

A JNK inhibitor according to the present invention may thus for example comprise an inhibitory (poly-)peptide sequence according to any of SEQ ID NOs: 2 to 27 (see table 1).

Table 1:
Examples for inhibitory (poly-)peptide sequences of JNK-inhibitors according to the present invention

Amino acid sequence	SEQ ID NO:
rPKRPTTLNLF	2
RPkRPTTLNLF	3
RPKRPaTLNLF	4
RPKRPTTLnLF	5

RPKRPTTLrLF	6
RPKRPTTLNLf	7
RPkRPaTLNLf	8
RPkRPTTLNLf	9
RPkRPTTLrLf	10
RRrRPTTLNLf	11
QRrRPTTLNLf	12
RPkRPTTLNLw	13
RPkRPTDLNLf	14
RRrRPTTLrLw	15
QRrRPTTLrLw	16
RRrRPTDLrLw	17
QRrRPTDLrLw	18
RRrRPaTLNLf	19
QRrRPaTLNLf	20
RrKRPaTLNLf	21
RPkRPsTLNLf	22
RPkRPqTLNLf	23
RPkRPkTLNLf	24
rGKRKALKLf	25
rGKRKALrLf	26
RRrRKALrLf	27

The JNK inhibitor according to the present invention may also be a JNK inhibitor (variant) which comprises an inhibitory (poly-)peptide sequence sharing at least 50%, more preferably at least 55%, more preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, most preferably at least 90% sequence identity with a sequence selected from SEQ ID NOs: 1-27, in particular with SEQ ID NO: 8,

with the proviso that with regard to the respective sequence selected from SEQ ID NOs: 1-27, such inhibitory (poly-)peptide sequence sharing sequence identity

a) maintains the L-arginine (R) residue on position 4,

b) maintains the two L-leucine (L) residues at position 8 and 10 (positions 7 and 9 with regard to SEQ ID NOs: 25-27),

c) exhibits one, two, three, four, five or six D-amino acid(s) at the respective positions corresponding to the amino acids selected from the group consisting of X1, X2, X3, X5, X7 and X8 of SEQ ID NO: 1 and respective positions in SEQ ID NOs: 2-27, more preferably exhibits one, two, three or four D-amino acid(s) at the positions corresponding to the amino acids selected from the group consisting of X3, X5, X7 and X8 of SEQ ID NO: 1 and respective positions in SEQ ID NOs: 2-27, and

d) still inhibits JNK activity (i.e. is a JNK inhibitor as defined herein).

Certainly, variants disclosed herein (in particular JNK inhibitor variants comprising an inhibitory (poly-)peptide sequence sharing - within the above definition – a certain degree of sequence identity with a sequence selected from SEQ ID NOs: 1-27), share preferably less than 100% sequence identity with the respective reference sequence.

In view of said definition and for sake of clarity the residues which may not be changed in variants of JNK inhibitors comprising SEQ ID NOs: 1-27 (see a) and b) in the above definition) are underlined in table 1.

The non-identical amino acids are preferably the result of conservative amino acid substitutions.

Conservative amino acid substitutions, as used herein, may include amino acid residues within a group which have sufficiently similar physicochemical properties, so that a substitution between members of the group will preserve the biological activity of the molecule (see e.g. Grantham, R. (1974), Science *185*, 862-864). Particularly, conservative amino acid substitutions are preferably substitutions in which the amino acids originate from the same class of amino acids (e.g. basic amino acids, acidic amino acids, polar amino acids, amino acids with aliphatic side chains, amino acids with positively or negatively charged side chains, amino acids with aromatic groups in the side chains, amino acids the side chains of which can enter into hydrogen bridges, e.g. side chains which have a hydroxyl function, etc.). Conservative substitutions are in the present case for example substituting a basic amino acid residue (Lys, Arg, His) for another basic amino acid residue (Lys, Arg, His), substituting an aliphatic amino acid residue (Gly, Ala, Val, Leu, Ile) for another aliphatic amino acid residue, substituting an aromatic amino acid residue (Phe, Tyr, Trp) for another aromatic amino acid residue, substituting threonine by serine or leucine by isoleucine. Further conservative amino acid exchanges will be known to the person skilled in the art. The isomer form should preferably be maintained, e.g. K is preferably substituted for R or H, while k is preferably substituted for r and h.

Further possible substitutions within the above definition for JNK inhibitor variants are for example if:

a) one, two or more of X1, X2, X3, X4, X5, X6, X7 and/or X8 of SEQ ID NO: 1 or the corresponding positions within the respective sequence selected from SEQ ID NOs: 2-27 are substituted for A or a,

b) X1 or X8 of SEQ ID NO: 1 or the corresponding position within the respective sequence selected from SEQ ID NOs: 2-27 is deleted;

c) X5 of SEQ ID NO: 1 or the corresponding position within the respective sequence selected from SEQ ID NOs: 2-27 is E, Y, L, V, F or K;

d) X5 of SEQ ID NO: 1 or the corresponding position within the respective sequence selected from SEQ ID NOs: 2-27 is E, L, V, F or K; or

e) one, two or three of X1, X2, X3 of SEQ ID NO: 1 or the corresponding positions within the respective sequence selected from SEQ ID NOs: 2-27 are neutral amino acids.

As used herein, the term "% sequence identity", has to be understood as follows: Two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A % identity may then be determined over the whole length of each of the sequences being compared (so-called global alignment), that is particularly suitable for sequences of the same or similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length. In the above context, an amino acid sequence having a "sequence identity" of at least, for example, 95% to a query amino acid sequence, is intended to mean that the sequence of the subject amino acid sequence is identical to the query sequence except that the subject amino acid sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain an amino acid sequence having a sequence of at least 95% identity to a query amino acid sequence, up to 5% (5 of 100) of the amino acid residues in the subject sequence may be inserted or substituted with another amino acid or deleted. For purposes of determining sequence identity, the substitution of an L-amino acid for a D-amino acid (and vice versa) is considered to yield a non-identical residue, even if it is merely the D- (or L-isomer) of the very same amino acid.

Methods for comparing the identity and homology of two or more sequences are well known in the art. The percentage to which two sequences are identical can for example be determined by using a mathematical algorithm. A preferred, but not limiting, example of a mathematical algorithm which can be used is the algorithm of Karlin *et al.* (1993), PNAS USA, 90:5873-5877.

Such an algorithm is integrated in the BLAST family of programs, e.g. BLAST or NBLAST program (see also Altschul *et al.*, 1990, J. Mol. Biol. 215, 403-410 or Altschul *et al.* (1997), Nucleic Acids Res, 25:3389-3402), accessible through the home page of the NCBI at world wide web site ncbi.nlm.nih.gov) and FASTA (Pearson (1990), Methods Enzymol. *183*, 63-98; Pearson and Lipman (1988), Proc. Natl. Acad. Sci. U. S. A *85*, 2444-2448.). Sequences which are identical to other sequences to a certain extent can be identified by these programmes. Furthermore, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux *et al.*, 1984, Nucleic Acids Res., 387-395), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of (Smith and Waterman (1981), J. Mol. Biol. *147*, 195-197.) and finds the best single region of similarity between two sequences.

Certainly, the JNK inhibitor according to present invention may comprise - aside of the inhibitory (poly-)peptide sequence mentioned above – additional sequences, domains, labels (e.g. fluorescent or radioactive labels), epitopes etc. as long as the ability to inhibit JNK activity as defined herein is not lost. For example, the JNK inhibitor according to the present invention may also comprise a transporter sequence. A "transporter sequence" as used herein, is a (poly-)peptide sequence providing for translocation of the molecule it is attached to across biological membranes. Accordingly, a JNK inhibitor according to the present invention comprising a transporter sequence is preferably capable of translocating across biological membranes. Thus, such JNK inhibitor of the present invention may more readily enter a cell, a cellular subcompartiment and/or into the nucleus of a cell.

Said transporter sequence may be joined for example (e.g. directly) N-terminally or (e.g. directly) C-terminally to the inhibitory (poly-)peptide sequence of the JNK inhibitor. The transporter sequence and the inhibitory (poly-)peptide sequence may also be spaced apart, e.g. may be separated by intermediate sequences. It is also contemplated that the transporter sequence may be positioned entirely elsewhere in the JNK inhibitor molecule than the inhibitory (poly-)peptide sequence, in particular if the JNK inhibitor is a more complex molecule (e.g. comprising several domains, is a multimeric conjugate etc.). It is also contemplated that the transporter sequence and the inhibitory (poly-)peptide sequence may overlap as long as the JNK inhibitory activity is maintained. Examples for such overlap are given further below.

Transporter sequences for use with the JNK inhibitor of the present invention may be selected from, without being limited thereto, transporter sequences derived from HIV TAT (HIV), e.g. native proteins such as e.g. the TAT protein (e.g. as described in U.S. Patent Nos. 5,804,604 and

5,674,980, each of these references being incorporated herein by reference), HSV VP22 (*Herpes simplex*) (described in e.g. WO 97/05265; Elliott and O'Hare, Cell 88 : 223-233 (1997)), non-viral proteins (Jackson et al, Proc. Natl. Acad. Sci. USA 89 : 10691-10695 (1992)), transporter sequences derived from Antennapedia, particularly from *Drosophila antennapedia* (e.g. the antennapedia carrier sequence thereof), FGF, lactoferrin, etc. or derived from basic peptides, e.g. peptides having a length of 5 to 15 amino acids, preferably 10 to 12 amino acids and comprising at least 80 %, more preferably 85 % or even 90 % basic amino acids, such as e.g. arginine, lysine and/or histidine, or may be selected from e.g. arginine rich peptide sequences, such as RRRRRRRRR (R_9; SEQ ID NO: 152), RRRRRRRR (R_8; SEQ ID NO: 153), RRRRRRR (R_7; SEQ ID NO: 154), RRRRRR (R_6, SEQ ID NO: 155), RRRRR (R_5, SEQ ID NO: 156) etc., from VP22, from PTD-4 proteins or peptides, from RGD-K_{16}, from PEPT1/2 or PEPT1/2 proteins or peptides, from SynB3 or SynB3 proteins or peptides, from PC inhibitors, from P21 derived proteins or peptides, or from JNKI proteins or peptides.

Examples of transporter sequences for use in the JNK inhibitor of the present invention are in particular, without being limited thereto, basic transporter sequences derived from the HIV-1 TAT protein. Preferably, the basic transporter sequence of the HIV-1 TAT protein may include sequences from the human immunodeficiency virus HIV-1 TAT protein, e.g. as described in, e.g., U.S. Patent Nos. 5,804,604 and 5,674,980, each incorporated herein by reference. In this context, the full-length HIV-1 TAT protein has 86 amino acid residues encoded by two exons of the HIV TAT gene. TAT amino acids 1-72 are encoded by exon 1, whereas amino acids 73-86 are encoded by exon 2. The full-length TAT protein is characterized by a basic region which contains two lysines and six arginines (amino acids 49-57) and a cysteine-rich region which contains seven cysteine residues (amino acids 22-37). The basic region (i.e., amino acids 49-57) was thought to be important for nuclear localization. Ruben, S. *et al.*, J. Virol. 63: 1-8 (1989); Hauber, J. *et al.*, J. Virol. 63 1181-1187 (1989). The cysteine-rich region mediates the formation of metal-linked dimers in vitro (Frankel, A. D. et al, Science 240: 70-73 (1988); Frankel, A. D. *et al.*, Proc. Natl. Acad. Sci USA 85: 6297-6300 (1988)) and is essential for its activity as a transactivator (Garcia, J. A. *et al.*, EMBO J. 7: 3143 (1988); Sadaie, M. R. *et al.*, J. Virol. 63:1 (1989)). As in other regulatory proteins, the N-terminal region may be involved in protection against intracellular proteases (Bachmair, A. *et al.*, Cell 56: 1019-1032 (1989)). Preferred TAT transporter sequences for use in the JNK inhibitor of the present invention are preferably characterized by the presence of the TAT basic region amino acid sequence (amino acids 49-57 of naturally-occurring TAT protein); the absence of the TAT cysteine-rich region amino acid sequence (amino acids 22-36 of naturally-occurring TAT protein) and the absence of the TAT exon 2-encoded carboxy-terminal domain (amino acids 73-86 of naturally-occurring TAT protein). More preferably, the transporter sequence

in the JNK inhibitor of the present invention may be selected from an amino acid sequence containing TAT residues 48-57 or 49 to 57 or variants thereof.

Preferably, the transporter sequence in a given JNK inhibitor of the present invention also exhibits D-amino acids, for example in order to improve stability towards proteases. Particularly preferred are transporter sequences which exhibit a specific order of alternating D- and L-amino acids. Such order of alternating D- and L-amino acids (the motif) may follow –without being limited thereto - the pattern of any one of SEQ ID NOs: 28-30:

$d_lLLL_xd_mLLL_yd_n$ (SEQ ID NO: 28);

$dLLLd(LLLd)_a$ (SEQ ID NO: 29); and/or

dLLLdLLLd (SEQ ID NO: 30);

wherein:

d		is a D-amino acid;
L		is a L-amino acid;
a		is 0 – 3, preferably 0-2, more preferably 0, 1, 2 or 3, even more preferably 0, 1, or 2 and most preferably 1;
l, m and n		are independently from each other 1 or 2, preferably 1;
x and y		are independently from each other 0, 1 or 2, preferably 1.

Said order of D- and L-amino acids (motif) becomes relevant when the transporter sequence is synthesized, i.e. while the amino acid sequence (i.e. the type of side chain residues) remains unaltered, the respective isomers alternate. For example, a known transporter sequence derived from HIV TAT is RKKRRQRRR (SEQ ID NO: 43). Applying the D-/L amino acid order of SEQ ID NO: 30 thereto would yield rKKRrQRRr (SEQ ID NO: 46).

In a particular embodiment the transporter sequence of the JNK inhibitor of the present invention may comprise at least one sequence according to rXXXrXXXr (SEQ ID NO: 31), wherein:

r	represents an D-enantiomeric arginine;
X	is any L-amino acid (including glycine);

and wherein each X may be selected individually and independently of any other X within SEQ ID NO: 31. Preferably at least 4 out of said 6 X L-amino acids within SEQ ID NO: 31 are K or R. In another embodiment the JNK inhibitor according to the present invention comprises the transporter sequence $rX_1X_2X_3rX_4X_5X_6r$ (SEQ ID NO: 32), wherein X_1 is K, X_2 is K, X_3 is R and X_4, X_5, and X_6 are any L-amino acid (including glycine) selected independently from each other. Similarly, the transporter sequence of the JNK inhibitor according to the present invention may comprise the

sequence $rX_1X_2X_3rX_4X_5X_6r$ (SEQ ID NO: 33), wherein X_4 is Q, X_5 is R, X_6 is R and X_1, X_2, and X_3 are any L-amino acid (including glycine) selected independently from each other. The inventive JNK inhibitor may also comprise the sequence $rX_1X_2X_3rX_4X_5X_6r$ (SEQ ID NO: 34), wherein one, two, three, four, five or six X amino acid residues are chosen from the group consisting of: X_1 is K, X_2 is K, X_3 is R, X_4 is Q, X_5 is R, X_6 is R, while the remaining X amino acid residues not selected from above group may be any L-amino acid (including glycine) and are selected independently from each other. X_1 is then preferably Y and/or X_4 is preferably K or R.

Examples of transporter sequences for use in the inventive JNK inhibitor molecule may be selected, without being limited thereto, from sequences as given in table 2 below, (SEQ ID NOs: 31-170) or from any fragment or variant or chemically modified derivative thereof (preferably it retains the function of translocating across a biological membrane).

Table 2:
Examples for transporter (poly-)peptide sequences for use in the JNK-inhibitors according to the present invention

SEQUENCE/PEPTIDE NAME	SEQ ID NO	AA	SEQUENCE
r3 (generic)	31	9	rXXXrXXXr
r3 (generic; right half)	32	9	$rKKRrX_4X_5X_6r$
r3 (generic; left half)	33	9	$rX_1X_2X_3rQRRr$
r3 (generic; individual)	34	9	$rX_1X_2X_3rX_4X_5X_6r$
TAT (1-86)	35	86	MEPVDPRLEP WKHPGSQPKT ACTNCYCKKC CFHCQVCFIT KALGISYGRK KRRQRRRPPQ GSQTHQVSLS KQPTSQSRGD PTGPKE
TAT (37-72)	36	36	CFITKALGIS YGRKKRRQRR RPPQGSQTHQ VSLSKQ
TAT (37-58)	37	22	CFITKALGIS YGRKKRRQRR RP
TAT (38-58)GGC	38	24	FITKALGISY GRKKRRQRRR PGGC
TAT CGG(47-58)	39	15	CGGYGRKKRR QRRRP
TAT (47-58)GGC	40	15	YGRKKRRQRR RPGGC
TAT (1-72) Mut Cys/Ala 72	41	56	MEPVDPRLEP WKHPGSQPKT AFITKALGIS YGRKKRRQRR RPPQGSQTHQ VSLSKQ
L-TAT (s1a)	42	10	GRKKRRQRRR (NH_2-GRKKRRQRRR-COOH)
L-TAT (s1b)	43	9	RKKRRQRRR (NH_2-GRKKRRQRRR-COOH)
L-TAT (s1c)	44	11	YDRKKRRQRRR
D-TAT	45	9	rrrqrrkkr
r_3-L-TAT	46	9	rKKRrQRRr
r_3-L-TATi	47	9	rRRQrRKKr
βA-r_3-L-TAT	48	9	βA-rKKRrQRRr (βA: beta alanine)

βA-r_3-L-TATi	49	9	βA-rRRQrRKKr (βA: beta alanine)
FITC-βA-r_3-L-TAT	50	9	FITC-βA-rKKRrQRRr (βA: beta alanine)
FITC-βA-r_3-L-TATi	51	9	FITC-βA-rRRQrRKKr (βA: beta alanine)
TAT(s2-1)	52	9	rAKRrQRRr
TAT(s2-2)	53	9	rKARrQRRr
TAT(s2-3)	54	9	rKKArQRRr
TAT(s2-4)	55	9	rKKRrARRr
TAT(s2-5)	56	9	rKKRrQARr
TAT(s2-6)	57	9	rKKRrQRAr
TAT(s2-7)	58	9	rDKRrQRRr
TAT(s2-8)	59	9	rKDRrQRRr
TAT(s2-9)	60	9	rKKDrQRRr
TAT(s2-10)	61	9	rKKRrDRRr
TAT(s2-11)	62	9	rKKRrQDRr
TAT(s2-12)	63	9	rKKRrQRDr
TAT(s2-13)	64	9	rEKRrQRRr
TAT(s2-14)	65	9	rKERrQRRr
TAT(s2-15)	66	9	rKKErQRRr
TAT(s2-16)	67	9	rKKRrERRr
TAT(s2-17)	68	9	rKKRrQERr
TAT(s2-18)	69	9	rKKRrQREr
TAT(s2-19)	70	9	rFKRrQRRr
TAT(s2-20)	71	9	rKFRrQRRr
TAT(s2-21)	72	9	rKKFrQRRr
TAT(s2-22)	73	9	rKKRrFRRr
TAT(s2-23)	74	9	rKKRrQFRr
TAT(s2-24)	75	9	rKKRrQRFr
TAT(s2-25)	76	9	rRKRrQRRr
TAT(s2-26)	77	9	rKRRrQRRr
TAT(s2-27)	78	9	rKKKrQRRr
TAT(s2-28)	79	9	rKKRrRRRr
TAT(s2-29)	80	9	rKKRrQKRr
TAT(s2-30)	81	9	rKKRrQRKr
TAT(s2-31)	82	9	rHKRrQRRr
TAT(s2-32)	83	9	rKHRrQRRr

TAT(s2-33)	84	9	rKKHrQRRr
TAT(s2-34)	85	9	rKKRrHRRr
TAT(s2-35)	86	9	rKKRrQHRr
TAT(s2-36)	87	9	rKKRrQRHr
TAT(s2-37)	88	9	rIKRrQRRr
TAT(s2-38)	89	9	rKIRrQRRr
TAT(s2-39)	90	9	rKKIrQRRr
TAT(s2-40)	91	9	rKKRrIRRr
TAT(s2-41)	92	9	rKKRrQIRr
TAT(s2-42)	93	9	rKKRrQRIr
TAT(s2-43)	94	9	rLKRrQRRr
TAT(s2-44)	95	9	rKLRrQRRr
TAT(s2-45)	96	9	rKKLrQRRr
TAT(s2-46)	97	9	rKKRrLRRr
TAT(s2-47)	98	9	rKKRrQLRr
TAT(s2-48)	99	9	rKKRrQRLr
TAT(s2-49)	100	9	rMKRrQRRr
TAT(s2-50)	101	9	rKMRrQRRr
TAT(s2-51)	102	9	rKKMrQRRr
TAT(s2-52)	103	9	rKKRrMRRr
TAT(s2-53)	104	9	rKKRrQMRr
TAT(s2-54)	105	9	rKKRrQRMr
TAT(s2-55)	106	9	rNKRrQRRr
TAT(s2-56)	107	9	rKNRrQRRr
TAT(s2-57)	108	9	rKKNrQRRr
TAT(s2-58)	109	9	rKKRrNRRr
TAT(s2-59)	110	9	rKKRrQNRr
TAT(s2-60)	111	9	rKKRrQRNr
TAT(s2-61)	112	9	rQKRrQRRr
TAT(s2-62)	113	9	rKQRrQRRr
TAT(s2-63)	114	9	rKKQrQRRr
TAT(s2-64)	115	9	rKKRrKRRr
TAT(s2-65)	116	9	rKKRrQQRr
TAT(s2-66)	117	9	rKKRrQRQr
TAT(s2-67)	118	9	rSKRrQRRr

TAT(s2-68)	119	9	rKSRrQRRr
TAT(s2-69)	120	9	rKKSrQRRr
TAT(s2-70)	121	9	rKKRrSRRr
TAT(s2-71)	122	9	rKKRrQSRr
TAT(s2-72)	123	9	rKKRrQRSr
TAT(s2-73)	124	9	rTKRrQRRr
TAT(s2-74)	125	9	rKTRrQRRr
TAT(s2-75)	126	9	rKKTrQRRr
TAT(s2-76)	127	9	rKKRrTRRr
TAT(s2-77)	128	9	rKKRrQTRr
TAT(s2-78)	129	9	rKKRrQRTr
TAT(s2-79)	130	9	rVKRrQRRr
TAT(s2-80)	131	9	rKVRrQRRr
TAT(s2-81)	132	9	rKKVrQRRr
TAT(s2-82)	133	9	rKKRrVRRr
TAT(s2-83)	134	9	rKKRrQVRr
TAT(s2-84)	135	9	rKKRrQRVr
TAT(s2-85)	136	9	rWKRrQRRr
TAT(s2-86)	137	9	rKWRrQRRr
TAT(s2-87)	138	9	rKKWrQRRr
TAT(s2-88)	139	9	rKKRrWRRr
TAT(s2-89)	140	9	rKKRrQWRr
TAT(s2-90)	141	9	rKKRrQRWr
TAT(s2-91)	142	9	rYKRrQRRr
TAT(s2-92)	143	9	rKYRrQRRr
TAT(s2-93)	144	9	rKKYrQRRr
TAT(s2-94)	145	9	rKKRrYRRr
TAT(s2-95)	146	9	rKKRrQYRr
TAT(s2-96)	147	9	rKKRrQRYr
TAT(s2-97)	148	8	rKKRrQRr
TAT(s2-98)	149	9	rKKRrQRrK
TAT(s2-99)	150	9	rKKRrQRrR
r_3R_6	151	9	rRRRrRRRr
L-R_9	152	9	RRRRRRRRR
L-R_8	153	8	RRRRRRRR

L-R_7	154	7	RRRRRRR
L-R_6	155	6	RRRRRR
L-R_5	156	5	RRRRR
r_9	157	9	rrrrrrrrr
r_5R_4 (D/L)	158	9	rRrRrRrRr
r_5R_4 (DD/LL)	159	9	rrRRrrRRr
PTD-4	160	11	YARAAARQARA
PTD-4 (variant 1)	161	11	WARAAARQARA
PTD-4 (variant 2)	162	11	WARAQRAAARA
L-P1 Penetratin	163	16	RQVKVWFQNRRMKWKK
D-P1 Penetratin	164	16	KKWKMRRNQFWVKVQR
JNKI, bestfit	165	17	WKRAAARKARAMSLNLF
JNKI, bestfit (variant 1)	166	17	WKRAAARAARAMSLNLF
MDCK transcytose sequence	167	9	RYRGDLGRR
YKGL	168	4	YKGL
P1	169	4	RRTK
P66	170	4	RRPK

As mentioned above, transporter sequences may also be selected from fragments or variants of the above sequences of table 2 (with the proviso that such fragment or variant retain preferably the function to provide for translocation across biological membranes). In this specific context, variants and/or fragments of those transporter sequences preferably comprise a peptide sequence sharing at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 85%, preferably at least 90%, more preferably at least 95% and most preferably at least 99% sequence identity over the whole length of the sequence of such a transporter sequence as defined in Table 2. In this specific context, a "fragment" of a transporter sequence as defined in Table 2, is preferably to be understood as a truncated sequence thereof, i.e. an amino acid sequence, which is N-terminally, C-terminally and/or intrasequentially truncated compared to the amino acid sequence of the original sequence.

Furthermore, a "variant" of a transporter sequence or its fragment as defined above, is preferably to be understood as a sequence wherein the amino acid sequence of the variant differs from the original transporter sequence or a fragment thereof as defined herein in one or more mutation(s), such as one or more substituted, (or, if necessary, inserted and/or deleted) amino acid(s). Preferably, variants of such a transporter sequence as defined above have the same biological

function or specific activity compared to the respective original sequence, i.e. provide for transport, e.g. into cells or the nucleus. In this context, a variant of such a transporter sequence as defined above may for example comprise about 1 to 50, 1 to 20, more preferably 1 to 10 and most preferably 1 to 5, 4, 3, 2 or 1 amino acid alterations. Variants of such a transporter sequence as defined above may preferably comprise conservative amino acid substitutions. The concept of conservative amino acid substitutions is known in the art and has already been set out above for the JNK inhibitory (poly-)peptide sequence and applies here accordingly.

The length of a transporter sequence incorporated in the JNK inhibitor of the present invention may vary. It is contemplated that in some embodiments the transporter sequence of the JNK inhibitor according to the present invention is less than 150, less than 140, less than 130, less than 120, less than 110, less than 100, less than 90, less than 80, less than 70, less than 60, less than 50, less than 40, less than 30, less than 20, and/or less than 10 amino acids in length.

Whether a specific transporter sequence is still functional in the context of the JNK inhibitor according to the present invention may easily be determined by a person skilled in the art. For instance, the JNK inhibitor comprising a transporter domain may be fused to a label, e.g. a fluorescent protein such as GFP, a radioactive label, an enzyme, a fluorophore, an epitope etc. which can be readily detected in a cell. Then, the JNK inhibitor comprising the transporter sequence and the label is transfected into a cell or added to a culture supernatant and permeation of cell membranes can be monitored by using biophysical and biochemical standard methods (for example flow cytometry, (immuno)fluorescence microscopy etc.).

Specific examples of JNK inhibitors according to the present invention comprising a transporter sequence are given in table 3:

Table 3:
Examples for JNK inhibitors comprising an inhibitory (poly-)peptide sequence and a transporter sequence

Amino acid sequence	AA	SEQ ID NO:
rKKRrQRRrRPkRPTTLNLf	20	171
rKKRrQRRrRPkRPaTLNLf	20	172
rKKRrQRRrRPkRPTTLrLf	20	173
rKKRrQRRrRPTTLNLf	17	174
rKKRrQRrRPTTLNLf	16	175

rKKRrQRRrRPkRPTTLNLw	20	176
rKKRrQRRrRPkRPTDLNLf	20	177
rKKRrQRRrRPTTLrLw	17	178
rKKRrQRrRPTTLrLw	16	179
rKKRrQRRrRPTDLrLw	17	180
rKKRrQRrRPTDLrLw	16	181
rKKRrQRRrRPaTLNLf	17	182
rKKRrQRrRPaTLNLf	16	183
rKKRrQRrKRPaTLNLf	17	184
rKKRrQRRrRPkRPsTLNLf	20	185
rKKRrQRRrRPkRPqTLNLf	20	186
rKKRrQRRrRPkRPkTLNLf	20	187
rKKRrQRRrGKRKALKLf	18	188
rKKRrQRRrGKRKALrLf	18	189
rKKRrQRRrRKALrLf	16	190

As mentioned above, in a particular embodiment of the present invention the transporter sequence and the inhibitory (poly-)peptide sequence may overlap. In other words, the N-terminus of the transporter sequence may overlap with the C-terminus of the inhibitory (poly-)peptide sequence or the C-terminus of the transporter sequence may overlap with the N-terminus of the inhibitory (poly-)peptide sequence. The latter embodiment is particularly preferred. Preferably, the transporter sequence overlaps by one, two or three amino acid residues with the inhibitory (poly-)peptide sequence. In such scenario a given transporter sequence may overlap with SEQ ID NO:1 or the respective variants thereof at position 1 (X1), position 1 and 2 (X1, X2), positions 1, 2 and 3 (X1, X2, X3).

SEQ ID NOs: 174, 175, 178, 179, 180, 181, 182, 183, 184, 188, 189 and 190 are good examples for JNK inhibitors according to the present invention, wherein transporter sequence and the inhibitory (poly-)peptide sequence overlap, e.g. rKKRrQ*RRrRPTTLNLf* (SEQ ID NO: 174) is an overlap of SEQ ID NO: 46 (underlined) and SEQ ID NO: 11 (italics).

Certainly the JNK inhibitor according to the present invention may also be selected from JNK inhibitors, which are a variant of any one of the JNK inhibitors according to SEQ ID NOs: 171-190. Preferably, such variant shares at least 50%, more preferably at least 55%, more preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%,

most preferably at least 95% sequence identity with the sequence of SEQ ID NOs: 171-190, in particular with SEQ ID NO: 172,

with the proviso that with respect to the inhibitory (poly-)peptide sequence within said sequences of SEQ ID NOs: 171-190 (see for reference inhibitory (poly-)peptide sequence of SEQ ID NO: 1 and specific examples of SEQ ID NOs: 2-27)) such sequence sharing sequence identity

a) maintains the L-arginine (R) residue on position 4 within the inhibitory (poly-)peptide sequence,

b) maintains the two L-leucine (L) residues at position 8 and 10 (positions 7 and 9 with regard to SEQ ID NOs: 25-27) within the inhibitory (poly-)peptide sequence,

c) exhibits at least one, at least two, at least three, at least four, at least five or six D-amino acid(s) at the respective positions corresponding to the amino acids selected from the group consisting of X1, X2, X3, X5, X7 and or X8 of SEQ ID NO: 1 and respective positions in SEQ ID NOs: 2-27, more preferably exhibits at least one, at least two, at least three or four D-amino acid(s) at the positions corresponding to the amino acids selected from the group consisting of X3, X5, X7 and X8 of SEQ ID NO: 1 and respective positions in SEQ ID NOs: 2-27, and

d) still inhibits JNK activity (i.e. is a JNK inhibitor as defined herein).

In view of said definition and for sake of clarity the residues which may not be changed in variants of JNK inhibitors comprising SEQ ID NOs: 171-190 (see a) and b) in the above definition) are underlined in table 3.

The non-identical amino acids in the variants of JNK inhibitors comprising SEQ ID NOs: 171-190 are preferably the result of conservative amino acid substitutions (see above). Certainly, the further possible substitutions mentioned above are also contemplated for variants of JNK inhibitors comprising SEQ ID NOs: 171-190. Likewise, the present invention certainly also contemplates variants of any one of the JNK inhibitors according to SEQ ID NOs: 171-190, which deviate from the original sequence not or not exclusively in the inhibitory (poly-)peptide sequence, but exhibits variant residues in the transporter sequence. For variants and fragments of transporter sequences see in particular respective disclosure above.

As mentioned previously, the transporter sequence and the JNK inhibitory (poly)-peptide sequence of the JNK inhibitors according to the present invention need not necessarily be directly joined to each other. They may also be spaced apart, e.g. by intermediate (poly-)peptide sequences. Preferred intermediate sequences separating the inhibitory (poly-)peptide sequences and other (functional) sequences such as transporter sequences consist of short peptide sequences less than

10 amino acids in length like a hexaamer, a pentamer, a tetramer, a tripeptide or even only a dipeptide or a single amino acid residue. Particularly preferred intermediate sequence are one, two or more copies of di-proline, di-glycine, di-arginine and/or di-lysine, all either in L-amino acid form only, or in D-amino acid form only, or with mixed D- and L-amino acids. Certainly, other known peptide spacer sequences may be employed as well.

A particularly preferred JNK inhibitor according to the present invention comprises SEQ ID NO: 8 (or a sequence sharing sequence identity with SEQ ID NO: 8 with the scope and limitations defined further above) and a transporter sequence. The transporter sequence is preferably selected from any one of SEQ ID Nos: 31-170 or variants thereof as defined herein, even more preferably from any one of SEQ ID NOs: 31-34 and 46-151. A particularly preferred embodiment of a JNK inhibitor according to the present invention is a JNK inhibitor comprising SEQ ID NO: 8 and SEQ ID NO: 46 (or sequences sharing respective sequence identity thereto within the scope and limitations defined further above). A preferred example is a JNK inhibitor comprising the sequence of SEQ ID NO: 172 or respective variants thereof varying in the transporter sequence and/or the inhibitory (poly-)peptide sequence as defined herein.

In a further aspect the present invention relates to a JNK inhibitor comprising

a) an inhibitory (poly-)peptide comprising a sequence from the group of sequences consisting of RPTTLNLF (SEQ ID NO: 191), KRPTTLNLF (SEQ ID NO: 192), RRPTTLNLF and/or RPKRPTTLNLF (SEQ ID NO: 193), and

b) a transporter sequence, preferably a transporter sequence selected from the transporter sequences disclosed in table 2 or variants/fragments thereof, even more preferably selected from SEQ ID NOs: 31-34 and 46-151 or respective variants or fragments thereof.

The transporter sequence and the inhibitory (poly-)peptide sequence may overlap. Preferred transporter sequences for said embodiment of the invention are particularly the transporter sequence of SEQ ID NO: 46, preferably joined (e.g. directly) to the N-Terminus of the inhibitory (poly-)peptide sequence.

A JNK inhibitor of the present invention may also be a JNK inhibitor comprising or consisting of the sequence GRKKRRQRRRPPKRPTTLNLFPQVPRSQD (SEQ ID NO: 194), or the sequence GRKKRRQRRRPTTLNLFPQVPRSQD (SEQ ID NO: 195).

In a further aspect the present invention relates to a (poly-)peptide comprising a transporter sequence selected from the group of sequences consisting of rKKRrQRr (SEQ ID NO: 148), rKKRrQRrK (SEQ ID NO: 149), and/or rKKRrQRrR (SEQ ID NO: 150).

As used herein, comprising a certain sequence or a certain SEQ ID NO: usually implies that (at least) one copy of said sequence is present, e g. in the JNK inhibitor molecule. For example, one inhibitory (poly-)peptide sequence will usually suffice to achieve sufficient inhibition of JNK activity. However, the inventor certainly contemplate that the use of two or more copies of the respective sequence (e.g. two or more copies of an inhibitory (poly-)peptide sequence of different or same type and/or two or more copies of a transporter sequence of different or the same type) may also employed as long as the overall ability of the resulting molecule to inhibit JNK activity is not abolished (i.e. the respective molecule is still a JNK inhibitor as defined herein).

The inventive JNK inhibitors may be obtained or produced by methods well-known in the art, e.g. by chemical synthesis via solid-phase peptide synthesis using Fmoc (9-fluorenylmethyloxycarbonyl) strategy, i.e. by successive rounds of Fmoc deprotection and Fmoc-amino acid coupling cycles. A commercial service offering such peptide synthesis is provided by many companies, for example the company PolyPeptide (Straßbourg, France).

Antibodies

In a further aspect the present invention relates to the production of antibodies raised against the JNK inhibitors of the present invention, i.e. methods of producing antibodies recognizing the JNK inhibitors of the present invention. Methods for producing antibodies are extremely well known in the art.

Thus, the present invention relates also to a method of immunizing a non-human animal with a JNK inhibitor according to the present invention, the method comprising the following step:

- contacting (immunizing) a non-human animal suitable for antibody production,
  in particular a non-human mammal,
  more preferably an animal selected from goat and rodents such as mouse, rat, and rabbit
  with a JNK inhibitor of the present invention,

more preferably with a JNK inhibitor comprising or consisting of a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27.

As used herein "immunizing" is understood to be of non-therapeutic nature, since the JNK inhibitors according to the present invention are no pathogens (i.e. there is no need for therapy).

The present invention relates also to a method of producing an (polyclonal) antibody recognizing a JNK inhibitor according to the present invention, the method comprising the step of:

- Isolating from a non-human animal suitable for antibody production,
  in particular a non human mammal,
  more preferably an animal selected from goat and rodents such as mouse, rat, and rabbit,
  which has been contacted (immunized) previously with a JNK inhibitor of the present invention,
  more preferably with a JNK inhibitor comprising or consisting of a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27,
  an (polyclonal) antibody recognizing said JNK inhibitor.

The present invention relates also to a method of isolating a cell producing an antibody recognizing a JNK inhibitor according to the present invention, the method comprising the step of:

- Isolating from a non-human animal suitable for antibody production,
  in particular a non human mammal,
  more preferably an animal selected from goat and rodents such as mouse, rat, and rabbit,
  which has been contacted (immunized) previously with a JNK inhibitor of the present invention,
  more preferably with a JNK inhibitor comprising or consisting of a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27,
  a cell producing said antibody recognizing said JNK inhibitor, and
  optionally immortalizing said cell.

The present invention relates also to a method of producing a (monoclonal) antibody recognizing a JNK inhibitor according to the present invention, the method comprising the step of:
Isolating an antibody recognizing a JNK inhibitor of the present invention,

more preferably recognizing a JNK inhibitor consisting of a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27,

from the cell culture supernatant of a cell producing said antibody, the cell being optionally immortalized.

A person skilled in the art will understand, that the method of immunizing a non-human animal and the method of producing an (polyclonal) antibody as disclosed herein may be carried out consecutively. Similarly, the method of immunizing a non-human animal, the method of isolating a cell producing an antibody and the method of producing an (monoclonal) antibody may be combined.

In a further aspect the present invention relates to an antibody producible (and/or produced) with the methods according to the present invention for producing a polyclonal or monoclonal antibody, wherein the antibody recognizes at least one (poly-)peptide comprising or consisting of a sequence selected from any one of SEQ ID NOs: 1-27, but does preferably not (or at least to lesser extent, e.g. at least by one order of magnitude) recognize the essentially same (poly-)peptide with L-amino acids in place of the D-amino acids in the respective sequence stretch of SEQ ID NO: 1-27. Preferably, such antibody does recognize a JNK inhibitor of the present invention, but does (or at least to lesser extent, e.g. at least by one order of magnitude) not recognize a (poly-)peptide comprising the sequence RPKRPTTLNLF (SEQ ID NO: 193)). A particularly preferred antibody (monoclonal or polyclonal) does recognize a JNK inhibitor comprising the sequence of SEQ ID NO: 8 (for example a JNK inhibitor comprising the sequence of SEQ ID NO: 172), but does not (or at least to lesser extent, e.g. at least by one order of magnitude) recognize a (poly-)peptide comprising the very same sequence with L-amino acids in place of the D-amino acids. Particularly preferred are such polyclonal or monoclonal antibodies recognizing a (poly-)peptide comprising SEQ ID NO: 172, but not recognizing (or at least recognizing to lesser extent, e.g. at least by one order of magnitude) a (poly-)peptide comprising the sequence RKKRRQRRRRPKRPATLNLF (SEQ ID NO: 199).

The present invention also relates to a cell isolated according to the above specified method of isolating a cell producing an antibody recognizing a JNK inhibitor according to the present invention, wherein the cell produces an antibody which preferably recognizes at least one (poly-)peptide selected from any one of SEQ ID NOs: 1-27, but does not recognize the essentially same (poly-)peptide with L-amino acids in place of the D-amino acids in the sequence corresponding to SEQ ID NO: 1, (e.g. does recognize a (poly-)peptide comprising the sequence RPkRPaTLNLf (SEQ

ID NO: 8), but does not recognize (or at least to lesser extent, e.g. at least by one order of magnitude) a (poly-)peptide comprising the sequence RPKRPTTLNLF (SEQ ID NO: 193).

The present invention also contemplates generating antibodies against the specific transporter sequences, thereby allowing to identify for example JNK inhibitors as disclosed in table 3. Consequently, all aspects (monoclonal or polyclonal antibodies; methods of generating the same, cells producing the same etc.) discussed above for antibodies recognizing a JNK inhibitor of the present invention (in particular at least one (poly-)peptide comprising or consisting of a sequence selected from any one of SEQ ID NOs: 1-27) may also be applied in the context of (poly-)peptide comprising or consisting of a sequence selected from any one of SEQ ID NOs: 31-34 and 46-151. Certainly, the reference sequence which must not be recognized (or at least to lesser extent, e.g. by at least one order of magnitude) is in this scenario again the very same sequence however with L-amino acids in place of the D-amino acids in the respective transporter sequence stretch.

Methods for testing (monoclonal and/or polyclonal) antibodies for their binding affinities are well known in the art. One possibility among other is to characterize the binding affinity of an antibody by means of a sandwich ELISA by using the target peptide as well as negative controls (e.g. the same peptide with L-amino acids only). The ELISA limit can –without being limited thereto - be calculated on blank replicates as follows:

ELISA limit = average (negative control) + (3x standard deviation of negative control).

If the sample value is less or equal to the ELISA limit the tested antibody may be considered to have no affinity to the target peptide. If the sample value exceeds the ELISA limit the tested antibody may be considered to exhibit affinity to the target peptide. Moreover, the higher the sample value, the stronger is the affinity of the tested antibody for the target.

A commercial service offering production of monoclonal or polyclonal antibodies is for example Eurogentec (Seraing, Belgium).

All references cited herein are herewith incorporated by reference.

Examples

In the following, particular examples illustrating various embodiments and aspects of the invention are presented. However, the present invention shall not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those

described herein will become readily apparent to those skilled in the art from the foregoing description, accompanying figures and the examples below. All such modifications fall within the scope of the appended claims.

Example 1: Synthesis of JNK inhibitor SEQ ID NO: 172

As illustrative example, synthesis of the JNK inhibitor with SEQ ID NO: 172 is set out below. A person skilled in the art will know that said synthesis may also be used for and easily adapted to the synthesis of any other JNK inhibitor according to the present invention.

The JNK inhibitor with SEQ ID NO: 172 was manufactured by solid-phase peptide synthesis using the Fmoc (9-fluorenylmethyloxycarbonyl) strategy. The linker between the peptide and the resin was the Rink amide linker (p-[Fmoc-2,3-dimethoxybenzyl]-phenoxyacetic acid). The peptide was synthesized by successive Fmoc deprotection and Fmoc-amino acid coupling cycles. At the end of the synthesis, the completed peptide was cleaved by trifluoroacetic acid (TFA) directly to yield the crude C-terminal amide, which was then purified by preparative reverse phase HPLC. The purified fractions were pooled in a homogeneous batch that is treated by ion exchange chromatography to obtain its acetate salt. The peptide was then freeze-dried.

1.1 Solid Phase Synthesis of the Peptide

Except when noted, the manufacturing took place at room temperature (22°C ± 7°C) in an air-filtered environment. The scale of synthesis was 0.7 mmoles of the starting amino acid on the resin, for an expected yield of about 1g of purified peptide. Synthesis was performed manually in a 30–50 mL reactor equipped with a fritted disk with mechanical stirring and/or nitrogen bubbling.

1.2 Preparation of the resin

The p-methylbenzhydrylamide resin (MBHA-resin) was first washed with dichloromethane/ dimethylformamide/diisoproplyethylamine under nitrogen. The washed resin was then coupled to the Rink amide linker (p-[Fmox-2,4-dimethoxybenzyl]-phenoxyacetic acid) in PyBOB(benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate)/ diisopropyl-ethylamine/1-hydroxybenzotriazole to yield Fmoc-Rink amide-MBHA resin.

1.3 Coupling of Amino Acids

Amino acids were coupled to the resin using the following cycle:

The Fmoc-Rink amide-MBHA resin was deprotected by washing it in 35% (v/v) piperidine/dimethylformamide, followed by dimethylformamide. The deprotection reaction took approximately 16 minutes. Fmoc-protected amino acids (e.g., 2 eq of amino acid and HOBt (1-hydroxybenzotriazole) in dimethylformamide/dichloromethane (50/50) were added to the resin followed by addition of 2 eq of the coupling agent diisopropylcarbodiimide (DIC). The coupling reaction took from one hour to overnight depending upon the respective amino acid being added. Volumes were calculated on a basis of 0.5 mL/100mg of peptide-resin and adjusted after each cycle. After coupling, the resin was washed 3 times with DMF. Completeness of coupling was tested by the ninhydrin test (or Kaiser test 1) on primary amines and the chloranyl test 2 on secondary amines. On some occasions, the chloranyl test may be associated with a ninhydrin test as a security control. In case the coupling test indicated incompleteness of reaction, coupling was repeated with a lower excess (0.5-1 eq) of amino acid, PYBOP, HOBT in dimethylformamide/dichloromethane and diisopropylethylamine. Functionality of the resin was measured and generally 0.6–0.2 meq/g, depending on the original loading of the resin. After the last amino acid has been coupled, the peptide-resin was deprotected as usual and then washed 5 times with DCM before drying in an oven under vacuum at 30°C. After the peptide-resin had dried, the yield of the solid-phase synthesis was calculated as the ratio of the weight increase of the peptide resin compared to the theoretical weight increase calculated from the initial loading of the resin. The yield may be close to 100%.

1.4 Cleavage And Deprotection

The peptide was cleaved from the resin in a mixture of trifluoroacetic acid/1,2-ethanedthiol/ thioanisole/water/phenol (88/2.2/4.4/4.4/7 v/v), also called TFA/K reagent, for 4 hours at room temperature. The reaction volume was 1mL/100mg of peptide resin. During addition of the resin to the reagent, the mixture temperature was regulated to stay below 30°C.

1.5 Extraction of the peptide from the resin:

The peptide was extracted from the resin by filtration through a fritted disc. After concentration on a rotavapor to 1/3 of its volume, the peptide was precipitated by cold t-butyl methyl ether and filtered. The crude peptide was then dried under vacuum at 30°C.

1.6 Preparative HPLC Purification:

The crude peptide was then purified by reverse-phase HPLC to a purity of ≥95%. The purified fractions were concentrated on a rotavaporator and freeze-dried.

1.7 Ion Exchange Chromatography

The concentrated freeze-dried pools of purified peptide with the sequence of SEQ ID NO: 172 was dissolved in water and purified by ion exchange chromatography on Dowex acetate, 50–100 mesh resin.

The required starting reagents for the synthesis were :

	CAS Registry Number	Chemical Name	Molecular Weight
Fmoc-Rink amide linker	145069-56-3	p-[Fmoc-2,4-dimethoxybenzyl]-phenoxyacetic acid	539.6
Fmoc-D-Ala-OH, H_2O	79990-15-1	N-alpha-Fmoc-D-alanine	311.3
Fmoc-Arg(Pbf)-OH	154445-77-9	N-alpha-Fmoc-N [2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl]-arginine	648.8
Fmoc-D-Arg(Pbf)-OH	187618-60-6	N-alpha-Fmoc-N [2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl]-D-arginine	648.8
Fmoc-Asn(Trt)-OH	132388-59-1	N-alpha-Fmoc-N- -trityl-asparagine	596.7
Fmoc-Gln(Trt)-OH	132327-80-1	N-alpha-Fmoc-N- -trityl-glutamine	610.7
Fmoc-Leu-OH	35661-60-0	N-alpha-Fmoc-leucine	353.4
Fmoc-Lys(Boc)-OH	71989-26-9	N-alpha-Fmoc-N -Boc-lysine	468.5
Fmoc-D-Lys(Boc)-OH	143824-78-6	N-alpha-Fmoc-N -Boc-D-lysine	468.5
Fmoc-D-Phe-OH	86123-10-6	N-alpha-Fmoc-D-phenylalanine	387.4
Fmoc-Pro-OH	71989-31-6	N-alpha-Fmoc-proline	337.4
Fmoc-Thr(tBu)-OH	71989-35-0	N-alpha-Fmoc-O-t-butyl-threonine	397.5

Other JNK inhibitors of the present invention may prepared in similar manner.

Example 2: Inhibitory efficacy of selected JNK inhibitors according to the present invention

In the following the standard operating procedure will be set forth describing how the Inhibitory efficacy of JNK inhibitors according to the present invention was measured. The method allows to measure in vitro, in a non radioactive standardized assay, the ability of a candidate compound to decrease the phosphorylation of the c-Jun specific substrate by JNK. Moreover, it will be illustrated how to determine the inhibitory effect (IC50) and the Ki of a chosen compound for JNK. The method is suitable to verify whether a candidate compound does or does not inhibit JNK activity. and a person skilled in the art will certainly understand how to adapt the below methods for his specific purposes and needs.

2.1 Material

AlphaScreen reagent and plate:

- His-JNK1 (ref 14-327, Upstate, 10 µg in 100 µl: concentration: 2.2 µM) 5nM final
- His-JNK2 (ref 14-329, Upstate, 10 µg in 100 µl: concentration: 2 µM) 5nM final
- His-JNK3 (ref 14-501, Upstate, 10 µg in 100 µl: concentration: 1.88 µM) 5nM final
- Anti-Phospho-cJun (ref 06-828, Upstate, lot DAM1503356, concentration: 44.5 µM) 10nM final
- Biotin-cJun (29-67):
sequence: Biotin – SNPKILKQSMTLNLADPVGSLKPHLRAKNSDLLTSPDVG (SEQ ID NO: 198), lot 100509 (mw 4382.11, P 99.28%) dissolved in H_2O, concentration: 10 mM) 30nM final
- ATP (ref AS001A, Invitrogen, lot 50860B, concentration 100 mM)) 5 µM final
- SAD beads (ref 6760617M, PerkinElmer, lot 540-460-A, concentration 5mg/ml) 20 µg/ml final
- AprotA beads (ref 6760617M, PerkinElmer, lot 540-460-A, concentration 5mg/ml) 20 µg/ml final
- Optiplate 384well white plate (ref 6007299, PerkinElmer, lot 654280/2008)
- 96well plate for peptide dilution (ref 82.1581, Sarstedt)

- TopSeals-A (ref 6005185, Perkin Elmer, Lot 65673)
- Bioluminescent energy transfer reading

- The bioluminescent energy transfer was read on the Fusion Alpha Plate reader (Perkin Elmer).

Pipette:

- An electronic EDP3 pipette 20-300 (Ref 17007243; Rainin) was used to fill in the plate with the Enzme-Antibody mix, the Subtrate-ATP mix and the Beads.
- A PIPETMAN® Ultra multichannel 8X20 (Ref 21040; Gilson) was used to fill in the plate with the inhibitory compounds.

Buffer and solutions

- Kinase Buffer: 20mM Tris-base pH 7.4, 10mM $MgCl_2$, 1mM DTT, 100µM Na_3VO_4, 0.01% Tween, (1% DMSO)
- Stop Buffer: 20mM Tris-base pH 7.4, 200mM NaCl, 80mM EDTA-K (pH de 8 with KOH instead of NaOH), 0.3% BSA
- JNK dilution Kinase buffer: 50mM Tris-base pH 7.4, 150mM NaCl, 0.1mM EGTA, 0.03% Brij-35, 270mM sucrose, 0.1% β–mercaptoethanol.

2.2 Method

To assess inhibitory effect of the peptides, a standard AlphaScreen assay (see for example Guenat et al. J Biomol Screen, 2006; 11: pages 1015-1026) was performed. The different components were prepared and subsequently mixed as indicated. The plates were sealed and incubated as following:

5 µl	JNK + Antibody	
5 µl	TP kinase + / - inhibiteur	Pre-incubation 30 min
5 µl	Biotin-cJun + ATP	Incubation 60 min at 24°C
10 µl	Beads SAD + A protA	Incubation 60 min in the dark at 24°C

To avoid contamination, the mixes were added with the pipette in different corner of the well. After the filling in of the plate with each mix, the plate was tapped (Keep one side fix and let the opposite side tap the table) to let the mix go down the walls of the wells.

The bioluminescent energy transfer was read on the Fusion Alpha Plate reader (Perkin Elmer).

All compounds should at least be tested in triplicate in 3 independent experiments for each isoform of JNK. Possibly concentrations of the compounds to be tested were 0, 0.03 nM, 0.1 nM,

0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 µM, 3 µM, 10 µ M, 30 µM, and 100 µM. Controls were samples either without JNK or without substrate (c-Jun).

Mix preparation

JNK1, JNK2 and JNK3 5nM

Biotin-cJun 30 nM

ATP 5 µM; Anti phospho-cJun (S63) 10nM

Bille SAD/AprotA 20 µg/ml

Antibody [final] = 10nM (anti Phospho cJun (S63))

Detection part : [Mix] X5 (5 µl in final volume of 25 µl)

[Stock] = 44.5 µM (ref 06-828, Upstate, Lot DAM1503356)

10 nM → 50nM in Kinase Buffer

JNK1, JNK2 and JNK3 [final] = 5nM

Reaction part : [Mix] X3 (5 µl in final volume of 15 µl)

[Stock] = 2.2 µM for JNK1 (ref 14-327, Upstate, lot D7KN022CU)

2.0 µM for JNK2 (ref 14-329, Upstate, lot 33221CU)

1.88 µM for JNK3 (ref 14-501, Upstate, lot D7CN041CU)

5 nM → 15nM in Antibody Buffer

Inhibitor:

Reaction part : [Mix] X3 (5 µl in final volume of 15 µl)

[Stock] = 10 mM

100 µM	→	300 µM in Kinase Buffer
30 µM	→	90 µM in Kinase Buffer
10 µM	→	30 µM in Kinase Buffer
...		
0.03 nM	→	0.09 nM in Kinase Buffer
And 0 nM	→	Kinase Buffer

Two series of 10 times serial dilutions were performed in a 96 well plate, one beginning with 300 µM to 0 nM, the second with 90 µM to 0.03 nM. The peptides are added in the 384 plates with an 8 channels multipipette (ref F14401, Gilson, 8X20).

ATP [final] = 5 µM

Reaction part : [Mix] X3 (5 µl in final volume of 15 µl)
[Stock] = 100 mM (ref AS001A, Invitrogen, lot 50860B)
5 µM → 15 µM in Kinase Buffer

Biotin c-Jun [final] = 30nM
Reaction part : [Mix] X3 (5 µl in final volume of 15 µl)
[Stock] = 10 mM
30 nM → 30nM in ATP Buffer

Beads SAD / A ProtA [final] = 20 µg/ml (Light sensitive)
Detection part : [Mix] X 2.5 (10 µl in final volume of 25 µl)
[Stock] = 5 mg/ml → 20 µg/ml 50 µg/ml in STOP Buffer
Mix in the dark room (green Light) or in the darkness.

Analysis of the IC50 curves:
The analysis was performed by the GraphPad Prism4 software with the following equation: Sigmoidal dose-response (No constraint).
Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)))

The outliers data were avoided using Grugg's test.

Comparison of the IC50:
The analysis was performed by the GraphPad Prism4 software with the following test: One way ANOVA test followed by a Tukey's Multiple Comparison Test. $P<0.05$ was considerate as significant.

The Km of the ATP for JNK and the Km of biotin-cJun specific peptide were determined in the report AlphaScreen standardization assay

The mathematical relation between Ki and IC50 (Ki = IC50 / (1 + ([Substrate] / Km of the substrate)) may be used to calculate the Ki values.

Example 3: Internalization experiments and analysis

3.1 Materials and Methods for uptake experiments

a) Cell line:

The cell line used for this experiment was HL-60 (Ref CCL-240, ATCC, Lot 116523)

b) Culture medium and plates

RPMI (Ref 21875-091, Invitrogen, Lot 8296) or DMEM (Ref 41965, Invitrogen, Lot 13481) complemented on 05.05.2008 with:

10% FBS (Ref A64906-0098, PAA, Lot A15-151): decomplemented at 56°C, 30 min, on 04.04.2008.

1mM Sodium Pyruvate (Ref S8636, Sigma, Lot 56K2386)

Penicillin (100 unit/ml)/Streptomycin (100µg/ml) (Ref P4333, Sigma, Lot 106K2321)

PBS 10X (Ref 70011, Invitrogen, Lot 8277): diluted to 1X with sterile H_2O

Trypsine-0.05% EDTA (Ref L-11660, PAA, Lot L66007-1194)

6 well culture plates (Ref 140675, Nunc, Lot 102613)
24 well culture plates (Ref 142475, Nunc, Lot 095849)
96 well culture plates (Ref 167008, Nunc, Lot 083310)

96 well plates for protein dosing (Ref 82.1581, Sarstedt)

96 well plates for fluorescence measurement (Ref 6005279, Perkin Elmer)

c) Solutions

Poly-D-lysine coating solution (Sigma P9011 Lot 095K5104): 25µg/ml final diluted in PBS 1x

Acidic wash buffer: 0.2M Glycin, 0.15M NaCl, pH 3.0

Ripa lysis buffer: 10mM NaH_2PO_4 pH 7.2, 150mM NaCl, 1% Triton X-100, 1mM EDTA pH 8.0, 200µM Na_3VO_2, 0.1% SDS, 1X protease inhibitor cocktail (Ref 11873580001, Roche, Lot 13732700)

d) Microscopy and fluorescence plate reader

Cells were observed and counted using an inverted microscope (Axiovert 40 CFL; Zeiss; 20X).

The fluorescence was read with the Fusion Alpha Plate reader (Perkin Elmer).

e) Method

FITC marked peptide internalization was studied on suspension cells. Cells were plated into poly-DL-lysine coated dishes at a concentration of 1 x 10^6 cells/ml. Plates were then incubated for 24 h at 37 °C, 5 % CO_2 and 100% relative humidity prior to the addition of a known concentration of peptide. After peptide addition, the cells were incubated 30 min, 1, 6 or 24 h at 37 °C, 5 % CO_2 and 100 % relative humidity. Cells were then washed twice with an acidic buffer (Glycin 0.2 M, NaCl 0.15 M, pH 3.0) in order to remove the cell-surface adsorbed peptide (see Kameyama et al., (2007), *Biopolymers,* **88**, 98-107). The acidic buffer was used as peptides rich in basic amino acids adsorb strongly on the cell surfaces, which often results in overestimation of internalized peptide. The cell wash using an acidic buffer was thus employed to remove the cell-surface adsorbed peptides. The acid wash was carried out in determining cellular uptake of Fab/cell-permeating peptide conjugates, followed by two PBS washes. Cells were broken by the addition of the RIPA lysis buffer. The relative amount of internalized peptide was then determined by fluorescence after background subtraction and protein content normalization.

The steps are thus:

1. Cell culture
2. Acidic wash and cellular extracts
3. Analysis of peptide internalization with a fluorescence plate reader

f) Cell culture and peptide treatment

The 6 well culture plates are coated with 3 ml of Poly-D-Lys (Sigma P9011; 25 µg/ml in PBS), the 24 well plates with 600 µl and the 96 well plates with 125 µl and incubated for 4 h at 37°C, CO_2 5 % and 100 % relative humidity.

After 4 hours the dishes were washed twice with 3.5ml PBS, 700 µl or 150 µl PBS for the 6, 24 or 96 well plates, respectively.

The cells were plated into the dishes in 2.4 ml medium (RPMI) at plating densities of 1'000'000 cells/ml for suspension cells. After inoculation, the plates were incubated at 37°C, 5 % CO_2 and 100 % relative humidity for 24 hours prior to the addition of the peptide. Adherent cells should be at a density of 90-95% the day of treatment and were plated in DMEM :

well	Surface of culture	Medium	Nb adherent cells	Nb suspension cells

	(cm^2)			
96 well	0.3	100 – 200 µl	8'000 - 30'000	100'000
24 well	2	500 – 1000 µl	100'000 - 200'000	500'000- 1'000'000
35mm (P35) / 6 well	10	2,4 ml	250'000 - 2'100'000	2'400'000
60mm (P60)	20	3,5 ml	$15 * 10^5$	1'000'000/ml
10cm (P100)	60	10 ml	$15\text{-}60 * 10^5$	

The cells were treated with the desired concentration of FITC labeled peptide (stock solution at a concentration of 10 mM in H_2O).

Following peptide addition, the cells were incubated 0 to 24 hours (e.g. 30 min, 1, 6 or 24 hours) at 37 °C, CO_2 5 % and 100 % relative humidity.

Acidic wash and cellular extracts :

The extracts were cooled on ice.

Suspension cells (or cells, which don attach well to the dish):

Transfer the cells in « Falcon 15 ml ». To recover the maximum of cells, wash the dish with 1 ml of PBS.

Harvest the cells 2 min at 2400 rpm max.

Suspend the cells in 1 ml cold PBS.

Transfer the cells into a coated "Eppendorf tube" (coated with 1ml of poly D-Lys for 4hours and washed twice with 1ml PBS).

Wash three times with 1 ml of cold acidic wash buffer and centrifuge 2 min at 2400 rpm max.

Beware of the spreading of the cells in the "eppendorf".

Wash twice with 1 ml cold PBS to neutralize.

Add 50 µl of lysis RIPA Buffer.

Incubate 30 min-1h on ice with agitation.

Adherent cells:

Wash three times with 3 ml, 1 ml or 200 µl (for 6, 24 or 96 well plates, respectively) of cold acidic wash buffer. Beware of the cells who detach from the dish.

Wash twice with 1 ml cold PBS (for 6, 24 or 96 well plates, respectively) to neutralize.

Add 50 µl of lysis RIPA buffer.

Incubate 30 min-1h on ice with agitation.

Scrap the cells with a cold scrapper. The 24 and 96 well plates were directly centrifuged at 4000rpm at 4° for 15min to remove the cellular debris. Then the supernatants (100 or 50ml respectively for the 24 or 96 well plates) were directly transferred in a dark 96 well plated. The plates were read by a fluorescence plate reader (Fusion Alpha, Perkin Elmer).

Transfer the lysate in a coated "eppendorf" (coated with 1ml of poly D-Lys for 4hours and wash twice with 1ml PBS).

The lysed cells were then centrifuged 30 min at 10000 g at 4 °C to remove the cellular debris. Remove the supernatant and store it at -80 °C in a coated "Eppendorf tube" (coated with 1 ml of poly D-Lys for 4 hours and washed twice with 1 ml PBS).

Analysis of peptide internalization with a fluorescence plate reader :

The content of each protein extract was determined by a standard BCA assay (Kit N°23225, Pierce), following the instructions of the manufacturer.

The relative fluorescence of each sample is determined after reading 10 µl of each sample in a fluorescence plate reader (Fusion Alpha, Perkin Elmer), background subtraction and normalization by protein concentration.

3.2 Uptake experiments

The time dependant internalization (uptake) of FITC-labeled TAT derived transporter constructs into cells of the HL-60 cell line was carried out as described above using sequences transporter peptides of SEQ ID NOs: 52-96, 43, and 45-47. These sequences are listed below in Table 4.

Table 4:
Transporter sequence tested in uptake experiments

SEQ ID NO:	peptide No: abbreviation in Figure 6											
46	r3-L-TAT	H2N	dR	K	K	R	dR	Q	R	R	dR	CONH2
52	1	H2N	dR	A	K	R	dR	Q	R	R	dR	CONH2
53	2	H2N	dR	K	A	R	dR	Q	R	R	dR	CONH2
54	3	H2N	dR	K	K	A	dR	Q	R	R	dR	CONH2
55	4	H2N	dR	K	K	R	dR	A	R	R	dR	CONH2
56	5	H2N	dR	K	K	R	dR	Q	A	R	dR	CONH2
57	6	H2N	dR	K	K	R	dR	Q	R	A	dR	CONH2
58	7	H2N	dR	D	K	R	dR	Q	R	R	dR	CONH2
59	8	H2N	dR	K	D	R	dR	Q	R	R	dR	CONH2
60	9	H2N	dR	K	K	D	dR	Q	R	R	dR	CONH2
61	10	H2N	dR	K	K	R	dR	D	R	R	dR	CONH2
62	11	H2N	dR	K	K	R	dR	Q	D	R	dR	CONH2
63	12	H2N	dR	K	K	R	dR	Q	R	D	dR	CONH2
64	13	H2N	dR	E	K	R	dR	Q	R	R	dR	CONH2
65	14	H2N	dR	K	E	R	dR	Q	R	R	dR	CONH2
66	15	H2N	dR	K	K	E	dR	Q	R	R	dR	CONH2
67	16	H2N	dR	K	K	R	dR	E	R	R	dR	CONH2
68	17	H2N	dR	K	K	R	dR	Q	E	R	dR	CONH2
69	18	H2N	dR	K	K	R	dR	Q	R	E	dR	CONH2
70	19	H2N	dR	F	K	R	dR	Q	R	R	dR	CONH2

71	20	H2N	dR	K	F	R	dR	Q	R	R	dR	CONH2
72	21	H2N	dR	K	K	F	dR	Q	R	R	dR	CONH2
73	22	H2N	dR	K	K	R	dR	F	R	R	dR	CONH2
74	23	H2N	dR	K	K	R	dR	Q	F	R	dR	CONH2
75	24	H2N	dR	K	K	R	dR	Q	R	F	dR	CONH2
76	25	H2N	dR	R	K	R	dR	Q	R	R	dR	CONH2
77	26	H2N	dR	K	R	R	dR	Q	R	R	dR	CONH2
78	27	H2N	dR	K	K	K	dR	Q	R	R	dR	CONH2
79	28	H2N	dR	K	K	R	dR	R	R	R	dR	CONH2
80	29	H2N	dR	K	K	R	dR	Q	K	R	dR	CONH2
81	30	H2N	dR	K	K	R	dR	Q	R	K	dR	CONH2
82	31	H2N	dR	H	K	R	dR	Q	R	R	dR	CONH2
83	32	H2N	dR	K	H	R	dR	Q	R	R	dR	CONH2
84	33	H2N	dR	K	K	H	dR	Q	R	R	dR	CONH2
85	34	H2N	dR	K	K	R	dR	H	R	R	dR	CONH2
86	35	H2N	dR	K	K	R	dR	Q	H	R	dR	CONH2
87	36	H2N	dR	K	K	R	dR	Q	R	H	dR	CONH2
88	37	H2N	dR	I	K	R	dR	Q	R	R	dR	CONH2
89	38	H2N	dR	K	I	R	dR	Q	R	R	dR	CONH2
90	39	H2N	dR	K	K	I	dR	Q	R	R	dR	CONH2
91	40	H2N	dR	K	K	R	dR	I	R	R	dR	CONH2
92	41	H2N	dR	K	K	R	dR	Q	I	R	dR	CONH2
93	42	H2N	dR	K	K	R	dR	Q	R	I	dR	CONH2
94	43	H2N	dR	L	K	R	dR	Q	R	R	dR	CONH2
45	44 (D-TAT)	H2N	dR	dR	dR	dQ	dR	dR	dK	dK	dR	CONH2
47	45 (r3-L-TATi)	H2N	dR	R	R	Q	dR	R	K	K	dR	CONH2
46	46 (r3-L-TAT)	H2N	dR	K	K	R	dR	Q	R	R	dR	CONH2
43	47 (L-TAT)	H2N	R	K	K	R	R	Q	R	R	R	CONH2
99	48	H2N	dR	K	K	R	dR	Q	R	L	dR	CONH2
100	49	H2N	dR	M	K	R	dR	Q	R	R	dR	CONH2
101	50	H2N	dR	K	M	R	dR	Q	R	R	dR	CONH2
102	51	H2N	dR	K	K	M	dR	Q	R	R	dR	CONH2
103	52	H2N	dR	K	K	R	dR	M	R	R	dR	CONH2
104	53	H2N	dR	K	K	R	dR	Q	M	R	dR	CONH2
105	54	H2N	dR	K	K	R	dR	Q	R	M	dR	CONH2
106	55	H2N	dR	N	K	R	dR	Q	R	R	dR	CONH2
107	56	H2N	dR	K	N	R	dR	Q	R	R	dR	CONH2
108	57	H2N	dR	K	K	N	dR	Q	R	R	dR	CONH2
109	58	H2N	dR	K	K	R	dR	N	R	R	dR	CONH2
110	59	H2N	dR	K	K	R	dR	Q	N	R	dR	CONH2
111	60	H2N	dR	K	K	R	dR	Q	R	N	dR	CONH2
112	61	H2N	dR	Q	K	R	dR	Q	R	R	dR	CONH2
113	62	H2N	dR	K	Q	R	dR	Q	R	R	dR	CONH2
114	63	H2N	dR	K	K	Q	dR	Q	R	R	dR	CONH2
115	64	H2N	dR	K	K	R	dR	K	R	R	dR	CONH2
116	65	H2N	dR	K	K	R	dR	Q	Q	R	dR	CONH2
117	66	H2N	dR	K	K	R	dR	Q	R	Q	dR	CONH2
118	67	H2N	dR	S	K	R	dR	Q	R	R	dR	CONH2
119	68	H2N	dR	K	S	R	dR	Q	R	R	dR	CONH2
120	69	H2N	dR	K	K	S	dR	Q	R	R	dR	CONH2
121	70	H2N	dR	K	K	R	dR	S	R	R	dR	CONH2

122	71	H2N	dR	K	K	R	dR	Q	S	R	dR	CONH2
123	72	H2N	dR	K	K	R	dR	Q	R	S	dR	CONH2
124	73	H2N	dR	T	K	R	dR	Q	R	R	dR	CONH2
125	74	H2N	dR	K	T	R	dR	Q	R	R	dR	CONH2
126	75	H2N	dR	K	K	T	dR	Q	R	R	dR	CONH2
127	76	H2N	dR	K	K	R	dR	T	R	R	dR	CONH2
128	77	H2N	dR	K	K	R	dR	Q	T	R	dR	CONH2
129	78	H2N	dR	K	K	R	dR	Q	R	T	dR	CONH2
130	79	H2N	dR	V	K	R	dR	Q	R	R	dR	CONH2
131	80	H2N	dR	K	V	R	dR	Q	R	R	dR	CONH2
132	81	H2N	dR	K	K	V	dR	Q	R	R	dR	CONH2
133	82	H2N	dR	K	K	R	dR	V	R	R	dR	CONH2
134	83	H2N	dR	K	K	R	dR	Q	V	R	dR	CONH2
135	84	H2N	dR	K	K	R	dR	Q	R	V	dR	CONH2
136	85	H2N	dR	W	K	R	dR	Q	R	R	dR	CONH2
137	86	H2N	dR	K	W	R	dR	Q	R	R	dR	CONH2
138	87	H2N	dR	K	K	W	dR	Q	R	R	dR	CONH2
139	88	H2N	dR	K	K	R	dR	W	R	R	dR	CONH2
140	89	H2N	dR	K	K	R	dR	Q	W	R	dR	CONH2
141	90	H2N	dR	K	K	R	dR	Q	R	W	dR	CONH2
142	91	H2N	dR	Y	K	R	dR	Q	R	R	dR	CONH2
143	92	H2N	dR	K	Y	R	dR	Q	R	R	dR	CONH2
144	93	H2N	dR	K	K	Y	dR	Q	R	R	dR	CONH2
145	94	H2N	dR	K	K	R	dR	Y	R	R	dR	CONH2
146	95	H2N	dR	K	K	R	dR	Q	Y	R	dR	CONH2
147	96	H2N	dR	K	K	R	dR	Q	R	Y	dR	CONH2

In the above table D amino acids are indicated by a small "d" prior to the respective amino acid residue (e.g. dR = D-Arg).

For a few sequences synthesis failed in the first approach unfortunately due to technical reasons. These sequences are abbreviated in Figure 6 as 1, 2, 3, 4, 5, 6, 7, 8, 43, 52, 53, 54, 55, 56, 57, 85, 86, 87, 88, 89, and 90. However, the remaining sequences were used in the internalization experiments.

The results are shown in Figure 6.

As can be seen in Figure 6, after 24 hours incubation, all transporters with the consensus sequence rXXXrXXXr (SEQ ID NO: 31) showed a higher internalization capability than the L-TAT transporter (SEQ ID NO: 43). Hela cells were incubated 24hours in 96well plate with 10mM of the r3-L-TAT-derived transporters. The cells were then washed twice with an acidic buffer (0.2M Glycin, 0.15M NaCl, pH 3.0) and twice with PBS. Cells were broken by the addition of RIPA lysis buffer. The relative amount of internalized peptide was then determined by reading the fluorescence intensity (Fusion Alpha plate reader; PerkinElmer) of each extract followed by background subtraction

As can be seen in Figure 6, one positions appears to be critical for highest transporter activity and for improved kinetics of transport activity: Y in position 2 (peptide N°91 corresponding to SEQ ID NO: 142).

The conclusion of this experiment is as follows:

- After 24 hours incubation, all transporters with the consensus sequence rXXXrXXXr (SEQ ID NO: 31) (see Table 2 for a selection of possible sequences) showed a higher internalization capability than the L-TAT transporter (SEQ ID NO: 43) (Figure 6). Those results fully validate the consensus sequence rXXXrXXXr (SEQ ID NO: 31).
- One position is critical for highest transporter activity and (Figure 6): Y in position 2 (sequence 91 corresponding to SEQ ID NO: 142).

Accordingly, such TAT derived sequences as shown in Table 4 are preferred, which exhibit an Y in position 2, particularly when the sequence exhibits 9 aa and has the consensus sequence rXXXrXXXr (SEQ ID NO: 31).

Example 4: Measurement of cytokine and chemokine release

In the following the procedure will be set forth describing how the released amount of several human cytokines after ligand induced secretion from human cells (Blood, WBC, PBMC, purified primary lymphocytes, cell lines, ...) was measured.

The technique used is a Sandwich ELISA, which allows measuring the amount of antigen between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two antigenic sites capable of binding to antibody, since at least two antibodies act in the sandwich. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible. The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2 to 5 times more sensitive than direct or indirect).

The method may be used to determine the effect of the JNK inhibitors of the present invention in vitro/ cell culture. At non toxic doses, compound efficacy is indicated by the decrease of the

cytokine levels (the variation of optical density (absorbance at 450 nm)) as compared to non-treated samples and is monitored by ELISA. Results are express in ng/ml.

4.1 Material

- 96 well plate:
  for collecting the supernatants (Ref 82.1581, Sarstedt)
  for ELISA (F96 maxisorp, Ref 442404, Nunc)
- TopSeal-A: 96well microplate seals (Ref 600585, PerkinElmer).
- ELISA reagent
  Coating buffer ELISA: 0.1M NaCarbonate pH 9.5 (= 7.13g $NaHCO_3$ (ref 71627, Fluka) + 1.59g Na_2CO_3 (ref 71345, Fluka) in 1 litre H2O, pH to 9.5 with NaOH concentrated)
  Wash buffer ELISA: PBS 1X + 0.01% Tween20. Prepare 1 litre PBS 1X (PBS10X: ref 70011, GIBCO) and add 100ul of Tween20 (ref P1379, Sigma) slowly while mixing with magnetic agitator)
  Assay diluent: PBS 1X + 10% FBS (Ref A15-151, PAA, decomplemented at 56°C, 30 min).
  DAKO TMB (ref S1599, DAKO): commercial substrate solution
  Stop Solution: 1M H_3PO_4 (→ for 200ml = 177ml H_2O + 23ml H_3PO_4 85% (ref 345245, Aldrich).
- ELISA Kit (reagent for 20 plates)
  IFN-γ: Human IFN- ELISA set, BD OptEIA™ (ref 555142, DB).
  IL-1β: Human IL-1 ELISA set II, BD OptEIA™ (ref 557953, BD)
  IL-10 : Human IL-10 ELISA set II, BD OptEIA™ (ref 555157, DB).
  IL-12 : Human IL-12 (p70) ELISA set, BD OptEIA™ (ref 555183, DB).
  IL-15 : Human IL-15 ELISA Set, BD OptEIA™ (ref 559268, DB).
  IL-2: Human IL-2 ELISA set, BD OptEIA™ (ref 555190, DB).
  IL-4 : Human IL-4 ELISA set, BD OptEIA™ (ref 555194, DB).
  IL-5 : Human IL-5 ELISA set, BD OptEIA™ (ref 555202, DB).
  IL-6: Human IL-6 ELISA setl, BD OptEIA™ (ref 555220, DB).
  IL-8: Human IL-8 ELISA set, BD OptEIA™ (ref 555244, DB).
  MCP-1: Human MCP-1 ELISA set, BD OptEIA™ (ref 555179, BD)
  TNF-α: Kit human TNF ELISA set, BD OptEIA™ (ref 555212, DB).
- Absorbance reading: The absorbance was read on the Fusion Alpha Plate reader (Perkin Elmer).

- Repeating pipettes, digital pipettes or multichannel pipettes.

4.2 Method

Preparation of the samples

The samples are culture medium supernatant from cultured human cells (typically whole blood, WBC, PBMC, Purified subtype of WBC, cancerous cell lines). Remove any particulate material by centrifugation (400g 5min 4°C) and assay immediately or store samples at -20°C. Avoid repeated freeze-thaw cycles.

One hour before using, defrost the samples on ice and centrifuge them. At step 11, dilute the samples in assay diluent directly into the plate (add first assay diluent, then the samples and pipette up and down):

Preparation of Standard

After warming lyophilized standard to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized standard with the proposed volume of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix. After reconstitution, immediately aliquot standard stock in polypropylene vials at 50 µl per vial and freeze at -20°C for up to 6 months. If necessary, store at 2-8° C for up to 8 hours prior to aliquotting/freezing. Do not leave reconstituted standard at room temperature.

Immediately before use, prepare a ten point standard curve using 2-fold serial dilutions in reagent Diluent. A high standard of 4000 pg/ml is recommended.

Preparation of Detector Mix

One-step incubation of Biotin/SAv reagents. Add required volume of Detection Antibody to Assay Diluent. Within 15 minutes prior to use, add required quantity of Enzyme Reagent, vortex or mix well. For recommended dilutions, see lot-specific Instruction/Analysis Certificate. Discard any remaining Working Detector after use.

Coating with Capture Antibody

1. Coat the wells of a PVC microtiter plate with 100 µL per well of Capture Antibody diluted in Coating Buffer. For recommended antibody coating dilution, see lot-specific Instruction/Analysis Certificate.

2. Cover the plate with an adhesive plastic and incubate overnight at 4°C.
3. Remove the coating solution and wash the plate by filling the wells with 150µl wash buffer.
4. The solutions or washes are removed by flicking the plate over a sink.
5. Repeat the process two times for a total of three washes.
6. After the last wash, remove any remaining wash buffer by patting the plate on a paper towel.

Blocking

7. Block the remaining protein-binding sites in the coated wells by adding 100µl reagent Diluent per well.
8. Cover the plate with an adhesive plastic and incubate for 1 h at room temperature.
9. During the incubation, start preparing the standard.

Adding samples

10. Do one wash as in step 3 with 150µl of wash buffer. The plates are now ready for sample addition.
11. Add 50 µl of appropriately diluted samples in assay diluent to each well. For accurate quantitative results, always compare signal of unknown samples against those of a standard curve. Standards (triplicates) and blank must be run with each cytokine to ensure accuracy.
12. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature.

Incubation with Detection Antibody and Secondary Antibody

13. Wash the plate four times with 150µl wash buffer like step 3.
14. Add 50 µl of detector MIX (detection antibody +Secondary Streptavidin-HRP antibody in assay diluent) to each well at recommended dilutions (see lot-specific Instruction/Analysis Certificate).
15. Cover the plate with an adhesive plastic and incubate for 1 h at room temperature light protect.
16. Wash the plate six times with 150µl wash buffer as in step 3.

17. Add 50 µl DAKO TMB solution to each well, incubate for 15-20 min at room temperature, in the dark, not sealed.

18. Add 50 µl of stop solution to each well. Gently tap the plate to ensure thorough mixing.

19. Mix the plate 5min at 500rpm on a plate mixer.

20. Read the optical density at 450 nm. (Program: Cytokine_ELISA on Fusion Alpha Plate reader).

Data analysis

Average the triplicate readings for each standard control and each sample. Subtract the average zero standard optical density (O.D). Create a standard curve plotting the log of the cytokine concentration versus the log of the O.D and the best fit line can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. A standard curve should be generated for each set of samples assayed. The outliers data were avoided using Grugg's test. Then the data which weren't in the interval of two times the SD, were discard. The independent experiments are taken into account if the positive control showed data as previously observed. The independent experiments are pooled ($N > 3$).

The data are presented in pg/ml of cytokine release or in %, compared to the induced condition without inhibitor treatment.

Example 5: THP1 differentiation – stimulation for cytokine release

In the following the procedure will be set forth describing how cytokine production from human PMA differentiated THP1 cells challenged by LPS for 6h was induced in order to test the ability of JNK inhibitors of the present invention, in particular of a JNK inhibitor with SEQ ID NO: 172, to reduce stimulation-induced cytokine release. THP1 cells were stimulated ex-vivo by different ligands for the readout of cytokine release. At non toxic doses, JNK inhibitor efficacy is indicated by the decrease of the cytokine levels as compared to non-treated samples and is monitored by ELISA. The toxicity of the compound are evaluated by the reduction of a tretazolium salt (MTS) to formazan, giving a purple colour.

Procedure:

a. Material

- Cell Line: THP-1 (Ref TIB-202, ATCC, lot 57731475)
- Culture medium, reagent and plates

RPMI (Ref 21875-091, Invitrogen) complemented with:

10% FBS (Ref A15-151, PAA): decomplemented at 56°C, 30 min.

10mM Hepes (Ref H0887, Sigma)

50 M -mercaptoethanol (Ref 63690, Fluka : stock at 14.3M): add 560 l of 50mM aliquots in PBS stocked at -20°C)

1mM Sodium Pyruvate (Ref S8636, Sigma)

Penicilline (100unit/ml) / Streptomycine (100 g/ml) (Ref P4333, Sigma)

The RPMI medium is then filtrated with a 0.22 M filter (Ref SCGPU05RE, Millipore).

PBS 10X (Ref 70011, Invitrogen): diluted to 1X with sterile H_2O

DMSO: Ref 41444, Fluka

PMA (phorbol 12-myristate 13-acetate, Ref P1585, Sigma, concentration 1mM = 616.8ug/ml in DMSO at -20°C). Use directly at a final concentration of 100nM in RPMI (1ul in 10ml of medium).

LPS ultrapure (Lipopolysaccharide, Ref tlrl-eklps, Invivogen, concentration 5mg/ml): Stock solution of LPS: 3 g/ml in PBS at 4°C. Use directly to prepare a 4X concentrated solution of 40ng/ml in RPMI medium (min 1800 l /plate; for 5 plates: 125 l of LPS 3 g/ml + 9250 l RPMI).

96 well plate:

for adherent cell culture (Ref 167008, Nunc)

for collecting the supernatants (Ref 82.1581, Sarstedt)

for ELISA (F96 maxisorp, Ref 442404, Nunc)

Coating solutions: poly-D-lysine (Ref P9011, Sigma): 25 g/ml final diluted in PBS 1x

- ELISA reagent and kits

Coating buffer ELISA: 0.1M NaCarbonate pH 9.5 (= 7.13g $NaHCO_3$ (ref 71627, Fluka) + 1.59g Na_2CO_3 (ref 71345, Fluka) in 1 liter H2O, pH to 9.5 with NaOH concentrated)

Wash buffer ELISA: PBS 1X + 0.01% Tween20 (ref P1379, Sigma, lot 094K0052)(= prepare 1 liter PBS 1X and add 100ul of Tween20 slowly while mixing with magnetic agitator)

Assay diluent: PBS 1X + 10% FBS (Ref A15-151, PAA, decomplemented at 56°C, 30 min).

DAKO TMB (ref S1599, DAKO): commercial substrate solution

Stop Solution: 1M H_3PO_4 (→ for 200ml = 177ml H_2O + 23ml H_3PO_4 85% (ref 345245, Aldrich).

TNF- : Kit human TNF ELISA set, BD OptEIA (ref 555212, DB).

- Cytotoxicity measurement: CellTiter 96 reagent (ref G3581, Promega)
- Control compound: SP600125 (ref ALX-270-339-M025, Alexis, concentration: 20mM DMSO)
- Absorbance reading: The absorbance was read on the Fusion Alpha Plate reader (Perkin Elmer).
- Repeating pipettes, digital pipettes or multichannel pipettes.
- TopSeal-A: 96well microplate seals (Ref 600585, PerkinElmer).

b. Method

Well coating

The plates had been coated with 200 l of poly D-Lysine (1x) and incubated 2 hours at 37°C, CO_2 5% and 100% relative humidity.

Cell plating

After 2 hours the wells were washed twice with 200 l PBS 1X (use immediately or leave with 200 l of PBS 1X at 37°C till use, but no more than 3 days).

The cells were counted. The desired number of cells was taken and resuspended in the amount of media necessary to get a dilution of 1'000'000 cells/ml. 100nM of PMA was added to induce the differentiation of the THP1 from suspension monocytes to adherent macrophages. The cells were plated into the wells in 100 l medium at plating densities of 100'000cells/well. After inoculation, the plates were incubated at 37°C, 5% CO2 and 100% relative humidity 3 days to let them differentiate, prior to the addition of experimental drugs.

Cell treatment

After 3 days, the adherent cells were observed with the microscope. The media containing PMA was aspirated and replaced by 100 l of fresh RPMI media without PMA (no washing step with PBS 1X).

Experimental drug were prepared at the concentration of 10 mM in H_2O or DMSO and stored at -80°C. Prior to each daily use, one aliquot of JNK inhibitorwas defrost and diluted to reach a 4X concentrated solution (120 M) in RPMI medium and then to the desired concentration in RPMI. The SP600125 was diluted to reach a 4X concentrated solution (40 M) in RPMI medium and then to the desired concentration in RPMI containing 0.8% DMSO.

The plates were treated with 50 l of medium or a solution of 4X the final desired drug concentration (0, 100nM, 1, 3, 10 or 30 M final for JNK compound or at 0, 10, 100nM, 1, 3 or 10 M final for the SP600125 positive control). Following drug addition, the plates were incubated for an additional 1h at 37°C, 5% CO_2 and 100% relative humidity.

After 1hours, the secretion of TNF was induced by the addition of 50 l of a 4X concentrated dilution of LPS ultrapure (3ng/ml final).

Assay

After 6 hours, 100 l of the supernatant were transferred to new 96well plates. Those plates were sealed and stored at -20° till the analysis by ELISA (e.g. see example 4) of the secretion of the cytokines.

The cytotoxic effect of the compounds was evaluated by MTS absorbance (e.g. see example 4) and cells were observed using an inverted microscope (Axiovert 40 CFL; Zeiss; 10X).

Data analysis

Analyses of the data are performed as indicated in the ELISA (see example 4). Briefly, for ELISA: Average the triplicate readings for each standard control and each sample. Subtract the average zero standard optical density (O.D). Create a standard curve plotting the log of the cytokine concentration versus the log of the O.D and the best fit line can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. A standard curve should be generated for each set of samples assayed. The outliers data were avoid using Grugg's test. Then the data which weren't in the interval of two times the SD, were discard. The independent experiments are taken into account if the positive control showed data as previously observed. The independent experiments are pooled ($N > 3$).

For the Cytotoxicity effect evaluation: on each plate of each independent experiment taken into account for the cytokine release experiment analysis, the average of the absorbance of the medium alone was considerate as the background and subtracted to each absorbance value. The average of triplicate of the non treated cells of each compound was considerate as the 100% viability. The average of triplicate of each compound was normalized by its 100%. The outliers data were avoid using Grugg's test. Then the data which weren't in the interval of two times the SD, were discard. The independent experiments are pooled ($N > 3$).

All statistical comparisons of conditions were performed by the GraphPad Prism4 software with the following test: One way ANOVA test followed by a Tukey's Multiple Comparison Test. $P<0.05$ was considerate as significant..

Example 6: JNK inhibitor of SEQ ID NO: 172 and TNFα release in Primary Rat or human whole blood cells

Whole blood is collected from anesthetized rat or human healthy volunteers using a venipuncture connected to a pre-labeled vacuum tube containing sodium citrate. Tubes are gently mixed by inversion 7-8 times; and are then kept at RT until stimulation. JNK inhibitor of SEQ ID NO: 172_is prepared 6 times concentrated in PBS, and 30 µl/well of mix is added into 96-well plate. Whole blood is diluted by 1:2 in PBS and 120 µl of diluted blood is added in each well where either PBS alone or JNK inhibitor of SEQ ID NO: 172 has been previously added. Whole blood is incubated at 37°C; 85 rpm (Stuart Orbital incubator SI500) for 60 min. Activators (LPS) are the prepared, 30µl/well of LPS, 6 times concentrated. After 60min incubation, LPS is added to the blood, blood is mixed by pipetting up and down, and then kept for 4h under agitation (85rpm), at 37°C. After the 4h incubation, the plates are centrifuged at about 770g, 4°c for 15 min in a pre-cooled centrifuge. Supernatants are finally collected and kept at -20°c until cytokine measurement. Cytokine (IL-6, IL-2, IFNγ and TNFα) were then measured using standard Elisa kits (e.g. from R&D Systems: DuoSet Elisas; or from BD Biosciences: BD Opteia Set Elisa). Results are expressed as pg/ml of supernatant of the measured cytokine.

A similar experiment was conducted with PMA+ionomycin instead of LPS as activator/stimulant.

Example 7: Half-life of specific JNK inhibitors disclosed herein

The JNK inhibitors with the sequence of SEQ ID NOs: 196, 197, and 172 (0.1mM final concentration) were digested in human serum (10 and 50% in PBS 1x). The experiment was performed as described by Tugyi et al. (Proc Natl Acad Sci U S A, 2005, 413-418). . The remaining intact peptide was quantified by UPLC-MS. Stability was assessed for SEQ ID NOs: 196, 197, and 172 identically but in two separate assays. While the JNK inhibitor with SEQ ID NO: 196 was totally degraded into amino acids residues within 6 hours, the JNK inhibitor with SEQ ID NO: 172

was completely degraded only after 14 days. The JNK inhibitor with SEQ ID NO: 197 was still stable after 30 days.

Example 8: Dose-dependent inhibition by JNK inhibitor with sequence of SEQ ID NO: 172 of CD3/CD28-induced IL-2 release in rat primary T-cells

Control animal were sacrificed, lymph nodes (LN) were harvested and kept in complete RPMI medium. LN were smashed with complete RPMI on 70µm filter using a 5ml piston. A few drops of media were added to keep strainer wet. Cells were centrifuged for 7 min at 450g and 4°c. Pellet was resuspended in 5 ml fresh medium. Cells were passed again through cell strainer. An aliquot of cells was counted, while cells were centrifuged again 10min at 1400 rpm and 4°c. Cells were resupended in MACS buffer (80µl of MACS buffer per 10^7 cells). 10µl of anti-rat MHC microbeads were added per 10 million cells, cells were incubated for 15min at 4°-8°c. Cells were washed with 15ml MACS buffer and centrifuge for 7 min at 700g and 4°C. Pellet was resuspended in 500µl MACS buffer per 10^8 cells. One LS column was placed in the magnetic field of the MACS separator per animal. Column was first rinsed with 3 ml of MACS buffer. One tube was placed below the column in ice to collect cells = T cells (negative selection so we collect what is eluted). Cell suspension was added and elute was collected on ice. Column was washed 3 times with 3mL MACS buffer. Eluted T cells were centrifuges for 7 min at 700g and 4°C. Resuspended cells were counted and plated at density of 200000cells/well in 100µl of complete medium. Plates were precoated the day before experiment with 2µg/mL of CD3 antibody, and the day of experiment plates were washed three times with PBS. Cells were treated with 100µl of (poly-)peptide JNK inhibitor (SEQ ID NO: 172), two times concentrated for 1h before ligand activation. After 1h of pretreatment with (poly-)peptide JNK inhibitor (SEQ ID NO: 172), cells were then stimulated with 2µg/mL of anti CD28 antibody for 24h. After 24h of stimulation, supernatant were collected and stored at -20°C until analysis. Cytokines were then measured using standard Elisa kits. Results are expressed as pg/ml of supernatant of the measured cytokine.

In a further experiment, essentially the same protocol as set forth above was used, but in addition to the (poly-)peptide JNK inhibitors with SEQ ID NO: 172, JNK inhibitors with the sequence of SEQ ID NO: 197 and the drug molecule SP600125 were also tested thus allowing to compare the effects of these inhibitors on the inhibition of CD3/CD28-induced IL-2 release.

Example 9: JNK inhibitor and TNFα/IL-2 release in human whole blood:

Whole blood from human healthy volunteers was collected using a venipuncture connected to a pre-labeled vacuum tube containing sodium citrate. Tubes are gently mixed by inversion 7-8 times; and are then kept at RT until stimulation. 350µl of RPMI + P/S were added into 1,2 ml-96-well plate. 10 times concentrated of SEQ ID NO: 172 was prepared in RPMI+P/S (50µl per well). 50µl was added into 1.2ml- 96 well plates. 50µl of whole blood was then added in each well where either medium alone or JNK inhibitor has been previously added. Whole blood was incubated at 37°C, 5% CO2 for 60 min. 50µl / well of ligands diluted in RPMI+ P/S was prepared, corresponding to the final dilution 10 times concentrated. After 60min of incubation, ligand was added; wells were then mixed by pipetting up and down the blood. Whole blood was incubated for 3 days at 37°C (wells were mixed by pipetting each well up and down once per day). At the end of incubation, plates were mixed and then centrifuged at 2500rpm, 4°C for 15 min in a pre-cooled centrifuge. Cytokine were then measured using standard Elisa kits. Results are expressed as pg/ml of supernatant of the measured cytokine.

A similar experiment was carried out with slight modifications. In the case of CD3/CD8 stimulation, CD3 antibody was coated at 2µg/mL in PBS overnight at 4°C. The day of experiment, wells were washed three times with PBS and left in PBS until use at 37°C. CD28 antibody was added 1h after SEQ ID NO: 172 at final concentration of 2µg/mL; supernatants were collected after 3 days of stimulation.

Claims

1. JNK inhibitor, selected from the group consisting of:

a) a JNK inhibitor, which comprises an inhibitory (poly-)peptide sequence according to the following general formula:

X1-X2-X3-R-X4-X5-X6-L-X7-L-X8 (SEQ ID NO: 1),

wherein X1 is an amino acid selected from amino acids R, P, Q and r,
wherein X2 is an amino acid selected from amino acids R, P, G and r,
wherein X3 is an amino acid selected from amino acids K, R, k and r,
wherein X4 is an amino acid selected from amino acids P and K,
wherein X5 is an amino acid selected from amino acids T, a, s, q, k or is absent,
wherein X6 is an amino acid selected from amino acids T, D and A,
wherein X7 is an amino acid selected from amino acids N, n, r and K; and
wherein X8 is an amino acid selected from F, f and w, and
wherein an amino acid residue given in capital letters indicates an L-amino acid, while an amino acid residue given in small letters indicates a D amino acid residue,

with the proviso that at least one of the amino acids selected from the group consisting of X1, X2, X3, X5, X7 and X8 is/are a D-amino acid(s), and

b) a JNK inhibitor which comprises an inhibitory (poly-)peptide sequence sharing at least at least 80% sequence identity with SEQ ID NO: 1 as defined in a),
with the proviso that with respect to SEQ ID NO: 1 such inhibitory (poly-)peptide sequence sharing sequence identity with SEQ ID NO: 1 maintains the L-arginine (R) residue of SEQ ID NO: 1 at position 4 and the two L-leucine (L) residues of SEQ ID NO: 1 at positions 8 and 10 and that at least one of the remaining amino acids in said sequence sharing at least at least 80% sequence identity with SEQ ID NO: 1 is a D-amino acid.

2. JNK inhibitor according to claim 1, wherein at least one of the amino acids selected from the group consisting of X3, X5, X7 and X8 is/are a D-amino acid(s).

3. JNK inhibitor according to claim 1 or 2, wherein the inhibitory (poly-)peptide sequence is selected from anyone of SEQ ID NOs: 2-27.

4. JNK inhibitor according to claim 1 or 2, wherein the JNK inhibitor comprises an inhibitory (poly-)peptide sequence sharing at least 80% sequence identity with a sequence selected from any one of SEQ ID NOs: 2-27.

5. JNK inhibitor according to anyone of the preceding claims, wherein the JNK inhibitor comprises SEQ ID NO: 8 or an inhibitory (poly-)peptide sequence sharing at least 80% sequence identity with SEQ ID NOs: 8.

6. JNK inhibitor according to anyone of the preceding claims, wherein the JNK inhibitor comprises a transporter sequence.

7. JNK inhibitor according to claim 6, wherein the inhibitory (poly-)peptide sequence and the transporter sequence overlap.

8. JNK inhibitor according to claim 6 or 7, wherein the transporter sequence comprises a sequence of alternating D- and L-amino acids according to anyone of SEQ ID NOs: 28-30.

9. JNK inhibitor according to anyone of claims 6-8, wherein said transporter sequence is selected from any one of SEQ ID NOs: 31-170.

10. JNK inhibitor according to anyone of claims 6-9, wherein said transporter sequence is selected from any one of SEQ ID NOs: 31-34, 46, 47 and 52-151.

11. JNK inhibitor according to anyone of claims 6-10, wherein said transporter sequence is positioned directly N-terminal or directly C-terminal of the inhibitory (poly-)peptide sequence.

12. JNK inhibitor according to anyone of claims 6-11, wherein the JNK inhibitor comprises

a) a sequence according to any one of SEQ ID NOs: 171-190, or

b) a sequence sharing at least 50% sequence identity with at least one of SEQ ID NOs: 171-190, with the proviso that said sequence sharing sequence identity anyone of SEQ ID NOs: 171-190:

i) maintains the L-arginine (R) residue on position 4 in its sequence stretch corresponding to SEQ ID NO: 1,

ii) maintains the two L-leucine (L) in its sequence stretch corresponding to SEQ ID NO: 1, and

iii) exhibits at least one D-amino acid at positions X1, X2, X3, X5, X7 or X8 in its sequence stretch corresponding to SEQ ID NO: 1.

13. JNK inhibitor according to anyone of claims 6-12, wherein the JNK inhibitor comprises

a) the sequence of SEQ ID NO: 172 or

b) a sequence sharing 50% sequence identity with SEQ ID NO: 172, with the proviso that said sequence sharing 50% sequence identity with SEQ ID NO: 172

i) maintains the L-arginine (R) residue on position 4 in its sequence stretch corresponding to SEQ ID NO: 1,

ii) maintains the two L-leucine (L) in its sequence stretch corresponding to SEQ ID NO: 1, and

iii) exhibits at least one D-amino acid at positions X1, X2, X3, X5, X7 or X8 in its sequence stretch corresponding to SEQ ID NO: 1.

14. JNK inhibitor comprising:

a) an inhibitory (poly-)peptide comprising a sequence selected from the group of sequences consisting of RPTTLNLF (SEQ ID NO: 191), KRPTTLNLF (SEQ ID NO: 192), RRPTTLNLF and/or RPKRPTTLNLF (SEQ ID NO: 193), and

b) a transporter sequence selected from SEQ ID NOs: 31-34 and 46-151.

15. JNK inhibitor comprising the sequence of SEQ ID NO: 194 or 195.

16. (Poly-)peptide comprising a transporter sequence selected from the group of sequences consisting of rKKRrQRr (SEQ ID NO: 148), rKKRrQRrK (SEQ ID NO: 149), and/or rKKRrQRrR (SEQ ID NO: 150).

17. Method of immunizing a non-human animal with a JNK inhibitor according to anyone of claims 1-14, the method comprising the following step:

- contacting (immunizing) a non-human animal suitable for antibody production, in particular a non-human mammal,

more preferably an animal selected from goat and rodents such as mouse, rat, and rabbit

with a JNK inhibitor according to anyone of claims 1-14,

more preferably with a JNK inhibitor comprising a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27.

18. Method of producing an (polyclonal) antibody recognizing a JNK inhibitor according to anyone of claims 1-14, the method comprising the step of:

- Isolating from a non-human animal suitable for antibody production,

in particular a non human mammal,

more preferably an animal selected from goat and rodents such as mouse, rat, and rabbit,

which has been contacted (immunized) previously with a JNK inhibitor according to anyone of claims 1-14,

more preferably with a JNK inhibitor consisting of a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27,

an (polyclonal) antibody recognizing said JNK inhibitor.

19. Method of isolating a cell producing an antibody recognizing a JNK inhibitor according to anyone of claims 1-14, the method comprising the step of:

- Isolating from a non-human animal suitable for antibody production,

in particular a non human mammal,

more preferably an animal selected from goat and rodents such as mouse, rat, and rabbit,

which has been contacted (immunized) previously with a JNK inhibitor according to anyone of claims 1-14,

more preferably with a JNK inhibitor consisting of a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27,

a cell producing said antibody recognizing said JNK inhibitor, and

optionally immortalizing said cell.

20. Method of producing a (monoclonal) antibody recognizing a JNK inhibitor according to anyone of claims 1-14, the method comprising the step of:

Isolating an antibody recognizing a JNK inhibitor according to anyone of claims 1-14,

more preferably recognizing a JNK inhibitor consisting of a (poly-)peptide having a sequence selected from any one of SEQ ID NOs: 1-27,

from the cell culture supernatant of a cell producing said antibody, the cell being optionally immortalized.

21. Antibody producible with anyone of the methods according to claim 18 or 20, wherein the antibody recognizes at least one (poly-)peptide selected from any one of SEQ ID NOs: 1-27, but does not recognize the essentially same (poly-)peptide with L-amino acids in place of the D-amino acids.

22. Cell producible with the method according to claim 19, wherein the cell produces an antibody according to claim 21.