Anticancer fusion protein

The invention relates to the field of therapeutic fusion proteins, in particular recombinant fusion proteins. More particularly, the invention relates to fusion proteins containing the fragment of a sequence of soluble human TRAIL protein in combination with the sequence of a short proapoptotic peptide, pharmaceutical compositions containing them, their use in therapy, particularly as anticancer agents, and to polynucleotide sequences encoding the fusion proteins, expression vectors containing the polynucleotide sequences, and host cells containing these expression vectors.

Apoptosis (programmed cell death) is a process that plays important role in preventing cancer and in the treatment of cancer by using agents that induce apoptosis of abnormal cancer cells.

Signaling for apoptosis may initiated from outside a cell (extrinsic or death receptor pathway) or from inside a cell (intrinsic or mitochondrial pathway).

Activation of extrinsic apoptosis pathways in human cancer cells requires binding of a ligand by cell death receptors to activate receptors. Upon binding of a ligand, activated receptors induce apoptosis signals.

Initiation of intrinsic apoptosis inside a cell by mitochondrial pathway may be initiated on different levels of apoptotic cascade to finally cause induction or restoration of functions of proapoptogenic proteins (cytochrome c, SmacDiablo, AIF, p53, Bcl2 proteins family including BH3 domain family), nucleic acids degradation or activation of caspases.

TRAIL protein belonging to the cytokines family (Tumour Necrosis Factor-Related Apoptosis Inducing Ligand), also known as Apo2L (Apo2-ligand), is a potent activator of apoptosis in tumour cells and in cells infected by viruses. TRAIL is a ligand naturally occurring in the body. TRAIL protein, its amino acid sequence, coding DNA sequences and protein expression systems were disclosed for the first time in EP0835305A1.

TRAIL protein exerts its anticancer activity binding to pro-apoptotic TRAIL surface receptors 1 and 2 (TRAIL-R1/R2) and subsequent activation of these receptors. These receptors, also known as DR4 and DR5 (death receptor 4 and death receptor 5), belong to the TNF receptor family and are overexpressed by different types of cancer cells. Activation of the receptors can induce external signaling pathway of apoptosis independent from suppressor gene p53, which by activated caspase-8 leads to the activation of executive caspases and thereby degradation of nucleic acids. Caspase-8 released upon TRAIL activation may also cause the release of Bid protein and thereby indirect activation of mitochondrial pathway, Bid protein being translocated to mitochondria, where it stimulates the release of cytochrome c, thus indirectly amplifying the apoptotic signal from death receptors.

TRAIL acts selectively on tumour cells essentially without inducing apoptosis in healthy cells, which are resistant to this protein. Therefore, the enormous potential of TRAIL was recognized as an anticancer agent acting on a wide range of different types of tumour cells, including hematologic malignancies and solid tumours, and at the same time not influencing normal cells and exerting potentially relatively small side effects.

TRAIL protein is a type II membrane protein having the length of 281 amino acids, and its extracellular region comprising amino acid residues 114-281 upon cleavage by proteases forms soluble sTRAIL molecule of 20 kDa size, which is also biologically active. Both forms of TRAIL and sTRAIL are capable of triggering apoptosis via interaction with TRAIL receptors present on target cells. Strong antitumour activity and very low systemic toxicity of soluble part of TRAIL molecule was demonstrated using cell lines tests. Also, human clinical studies on recombinant human soluble TRAIL (rhTRAIL) having aminoacid sequence corresponding to amino acids 114-281 of hTRAIL, known under the INN dulanermin, showed its good tolerance and absence of dose-limiting toxicity.

Recent studies show that TRAIL protein can have a form shorter than aminoacids 114 - 281, and that also in such form is able to bind with membrane receptors of DR family (*death receptors, DR1, DR2, DcR1, DcR2* and *OPG)* and induce apoptotic via these receptors (*F., FANG, A., WANG, S.,F., YANG, Antitumor activity of a novel recombinant mutant human tumor*

necrosis factor-related apoptosis-inducing ligand, Acta Pharmacologica Sinica 2005 Nov; 26 (11): 1373-1381).

Presently reported toxic effects of recombinant TRAIL protein on liver cells appear to be associated with the presence of modification, i.e. polyhistidine tags, untagged TRAIL showing no systemic toxicity.

However, in the course of further research and development, it appeared that many cancer cells also show primary or acquired resistance to TRAIL (see for example WO2007/022214). Although the mechanism of resistance to TRAIL is not fully understood, it is believed that it may manifest itself at different levels of by TRAIL-induced apoptosis pathway, ranging from the level of receptors on the cell surface to the executive caspases within the signalling pathway. This resistance limits the usefulness of TRAIL as an anticancer agent.

Furthermore, in clinical trials on patients the actual effectiveness of TRAIL as a monotherapy proved to be low. To overcome this low efficiency and the resistance of tumours to TRAIL, various combination therapies were designed with radio- and chemotherapeutic agents, which resulted in synergistic apoptotic effect. (WO2009/002947; A. Almasan and A. Ashkenazi, Cytokine Growth Factor Reviews 14 (2003) 337-348; RK Srivastava, Neoplasis, Vol 3, No 6, 2001, 535-546, Soria JC et al., J. Clin. Oncology, Vol 28, No 9 (2010), p. 1527-1533). The use of rhTRAIL for cancer treatment in combination with selected conventional chemotherapeutic agents (paclitaxel, carboplatin) and monoclonal anti-VEGF antibodies are described in WO2009/140469. However, such a combination necessarily implies well-known deficiencies of conventional chemotherapy or radiotherapy.

Constructed fusion protein containing sequences of an angiogenesis inhibitor vasostatin and TRAIL linked with a metalloprotease cleavage site linker was described as exhibiting apoptosis-inducing effect in tumor cells by A.I. Guo et al in Chinese Journal of Biochemistry and Molecular Biology 2008, vol. 24(10), 925-930.

Constructed fusion protein containing sequences Tumstatin183-230 of an angiogenesis inhibitor tumstatin and TRAIL114-281 was described as exhibiting

induction of apoptosis of pancreatic cancer cells by N.Ren et al in Academic Journal of Second Military Medical University 2008, vol. 28(5), 676-478.

US2005/244370 and corresponding WO2004/035794 disclose the construct of TRAIL95-281 as an effector domain linked by a peptide linker with extracellular part of another member of TNF family ligands CD40 as a cell surface binding domain. It is stated that activation of the construct is via binding of its CD40 part.

Moreover, the problem connected with TRAIL therapy has proved to be its low stability and rapid elimination from the body after administration.

Although many clinical cancer therapies are currently available, they are often insufficiently effective and have many well-known disadvantages, of which one of the most distressing and restricting the treatment are the lack of selectivity towards cancer cells, severe side effects and resistance - primary or acquired during treatment. Currently, a limited number of anticancer agents that are both effective and selective to cancer cells is known. Therefore, there remains an urgent and unmet need for new anticancer agents that would allow both to broaden the range of available agents and to find agents that are more effective (cytotoxic) and selective. There is also a need for new selective agents with increased stability and improved pharmacokinetics.

The present invention proposes a solution of this problem by providing new fusion proteins that contain a domain derived from TRAIL and a short effector peptide domain not including TRAIL fragments having intrinsic (intracellular) or extrinsic (extracellular) proapoptotic activity, that potentiates or complements the action of TRAIL. Moreover, it turned out that in many cases the fusion proteins of the invention show more potent activity than soluble TRAIL and its variants, including a fragment of the sequence, and in many cases also overcome the resistance to TRAIL. Moreover, the addition of an effector peptide results in prolonged half-life and increased retention of protein in the tumour and finally increases its efficiency.

Summary of Disclosure

In one embodiment, there is provided a fusion protein comprising: domain (a) which is the functional fragment of soluble hTRAIL protein sequence, wherein said functional fragment is capable of inducing apoptotic signal and begins with an amino acid at a position not lower than hTRAIL95, or a sequence having at least 70% homology thereto; and domain (b) which is the sequence of a pro-apoptotic effector peptide, which effects its pro-apoptotic action via intrinsic apoptosis pathway, wherein the sequence of domain (b) is attached at the C-terminus and/or the N-terminus of domain (a).

In one embodiment, there is provided a polynucleotide sequence, coding the fusion protein as defined herein.

In one embodiment, there is provided a pharmaceutical composition, comprising as an active ingredient the fusion protein as defined herein in combination with a pharmaceutically acceptable carrier.

In one embodiment, there is provided a use of the fusion protein as defined herein or the pharmaceutical composition as defined herein for the preparation of a medicament for the treatment of cancer diseases in a mammal, including human.

Description of Figures.

The invention will now be described in detail with reference to the Figures of the drawing.

Fig. 1 presents a schematic structure of fusion proteins of the invention according to Ex. 1, Ex.2, Ex. 3, Ex. 4 and Ex. 5.

Fig. 2 presents a schematic structure of fusion proteins of the invention according to Ex. 6, Ex. 7, Ex. 8, Ex. 9 and Ex. 10.

Fig. 3 presents a schematic structure of fusion proteins of the invention according to Ex. 11, Ex. 12, Ex. 13, Ex. 14 and Ex. 15.

Fig. 4 presents a schematic structure of fusion proteins of the invention according to Ex. 16, Ex. 17, Ex. 18, Ex. 19 and Ex. 20.

Fig. 5 presents a schematic structure of fusion proteins of the invention according to Ex.21, Ex.22, and Ex.23, as well as comparative fusion proteins of Ex.24, Ex.25 and Ex. 26.

Fig. 6 presents a schematic structure of fusion proteins of the invention according to Ex. 27, Ex. 28, Ex. 29, Ex. 30 and Ex. 31.

Fig. 7 presents a schematic structure of fusion proteins of the invention according to Ex. 32, Ex. 33, Ex. 34, Ex. 35 and Ex. 36.

Fig. 8 presents a schematic structure of fusion proteins of the invention according to Ex. 37, Ex. 38, Ex. 39, Ex. 40 and Ex. 41.

Fig. 9 presents a schematic structure of fusion proteins of the invention according to Ex. 42, Ex. 43, Ex. 44, Ex. 45 and Ex. 46.

Fig. 10 presents a schematic structure of fusion proteins of the invention according Ex. 47, Ex. 48, Ex. 49, Ex. 50 and Ex. 51.

Fig. 11 presents a schematic structure of fusion proteins of the invention according Ex. 52, Ex. 53, Ex. 54, and Ex. 55.

Fig. 12 presents tumour volume changes in time in SCID/NOD mice burdened with colon cancer Colo205, treated with fusion proteins of the invention compared to hTRAIL114-281.

Fig. 13 presents the tumour growth inhibition values in mice burdened with colon cancer Colo205, treated with fusion proteins of the invention on the 29th day of experiment compared to hTRAIL114-281.

Fig. 14 presents tumour volume changes in time in mice Crl:SHO-PrkdcscidHrhr burdened with human lung cancer NCI-H460, treated with fusion proteins of the invention, compared with hTRAIL114-281.

Fig. 15 presents the Tumour growth inhibition in mice burdened with human lung cancer NCI-H460, treated with fusion proteins of the invention on the 29th day of experiment, compared with hTRAIL114-281

Fig. 16 presents tumour volume changes in time in mice Crl:SHO-PrkdcscidHrhr burdened with human small cell lung cancer A549, treated with fusion proteins of the invention, compared with hTRAIL114-281.

Fig. 17 presents the Tumour growth inhibition in mice burdened with human small cell lung cancer A549, treated with fusion proteins of the invention on the 34th day of experiment, compared with hTRAIL114-281

Fig. 18 presents tumour volume changes in time in mice Crl:SHO-PrkdcscidHrhr burdened with human pancreatic carcinoma, epithelial-like cell line PANC-1, treated with fusion proteins of the invention, compared with hTRAIL114-281.

Fig. 19 presents the Tumour growth inhibition in mice burdened with human pancreatic carcinoma, epithelial-like cell line PANC-1, treated with fusion proteins of the invention on the 43th day of experiment, compared with hTRAIL114-281.

Fig. 20 shows circular dichroism spectra for fusion proteins of Ex. 1, Ex. 2, Ex. 14, Ex. 24, Ex. 51 and Ex. 42 and for rhTRAI114-281 expressed in specific ellipticity.

Detailed Description of the Invention

The invention relates to a fusion protein comprising:

domain (a) which is the functional fragment of a sequence of soluble hTRAIL protein, which fragment begins with an aminoacid at a position not lower than hTRAIL95 or a sequence having at least 70% homology thereto, and

domain (b) which is the sequence of a pro-apoptotic effector peptide, which effects its pro-apoptotic action via intrinsic apoptosis pathway, wherein the sequence of the domain (b) is attached at the C-terminus and/or N-terminus of domain (a).

The term "the functional soluble fragment of a sequence of soluble hTRAIL" should be understood as denoting any such fragment of soluble hTRAIL that is capable of inducing apoptotic signal.

It will be also appreciated by a skilled person that the existence of a 70% homology of the TRAIL is known in the art.

The term "a peptide" in accordance with the invention invention should be understood as a molecule built from plurality of aminoacids linked together through a peptide bond. Thus, the term "peptide" according to the invention includes oligopeptides, polypeptides and proteins.

It should be understood that domain (b) of the effector peptide in the fusion protein of the invention is neither hTRAIL protein nor a part of hTRAIL protein.

In the present invention the aminoacid sequences of peptides will be presented in a conventional manner adopted in the art in the direction from N-terminus (N-end) of the peptide towards its C-terminus (C-end). Any sequence will thus have its N-terminus on the left side and C-terminus on the right side.

The fusion protein of the invention may comprise a single domain (b) of the effector peptide, attached at the C-terminus or N-terminus of domain (a).

The fusion protein of the invention may also contain two domains (b) of the effector peptide, in which case one of the domains (b) is attached at the C-terminus of domain (a) and the other is attached at the N-terminus of domain (a).

When the fusion protein of the invention comprises two domains (b) of the effector peptide, these domains may be the same or different. Preferably, in this case, the domains (b) are different.

In a particular embodiment, the domain (a) is a fragment of hTRAIL sequence, beginning with an aminoacid from the range of hTRAIL114 to hTRAIL121, inclusive, and ending on the aminoacid hTRAIL 281, or other functional fragments of hTRAIL sequence published in GenBank under Accession No P50591.

In particular, domain (a) may be selected from the group consisting of sequences corresponding to hTRAIL114-281 (SEQ. No. 27), hTRAIL119-281 (SEQ. No. 28), and hTRAIL121-281 (SEQ. No. 29), hTRAIL116-281 and hTRAIL120-281.

In another embodiment, domain (a) may be the sequence hTRAIL95-281.

The pro-apoptotic effector peptide of domain (b), which exerts its apoptotic activity via intrinsic apoptosis pathway (intracellularly), may induce apoptosis directly by activating signalling cascade components of mitochondrial pathway of apoptosis, or by direct induction of mitochondrial apoptosis in cells.

In one embodiment of the fusion protein of the invention, the effector peptide is a peptide acting via intrinsic apoptosis pathway selected from the group consisting of SEQ. No. 30, No. 31, SEQ. No. 32, SEQ. No. 33, SEQ. No. 34, SEQ. No. 35, SEQ. No. 36, SEQ. No. 37, SEQ. No. 38, SEQ. No. 39, No. 40, SEQ. No. 41, SEQ. No. 42, SEQ. No. 43, SEQ. No. 44, SEQ. No. 45, SEQ. No. 46, and SEQ. No 47, or SEQ. No. 151, SEQ. No. 152, SEQ. No. 153, SEQ. No. 154, SEQ. No. 155, SEQ. No. 156, SEQ. No. 157, SEQ. No. 158 SEQ. No. 159, SEQ. No. 160, No. 161, SEQ. No. 162, SEQ. No. 163, SEQ. No. 164, SEQ. No. 165 and SEQ. No. 166.

The effector peptide of SEQ. No. 30 of the above group is the peptide derived from the BH3 domain of Bax protein which inhibits anti-apoptototic factors, and specifically the 16-amino acid peptide presented by:

KKLSECLKRI GDELDS SEQ. No. 30

It is believed that peptides based on sequences of BH3 domains of Bax protein are able to effectively bind to the anti-apoptotic proteins Bcl-2 and Bcl-XL. The anti-apoptotic activity of Bcl-2 and Bcl-XL protein is based on their interaction with BH3 domains present in factors responsible for the initiation of apoptosis (Bax, Bak, Bad). Binding of BH3 domain results in prevention of interaction of proteins Bcl-2 and Bcl-XL with their natural ligands and inhibition of their activity, and thereby contributes to initiation of the promotion of apoptosis.

The effector peptide of SEQ. No. 31 of the above group is the 15-amino acids peptide comprising the BH3domain of Bid protein, presented by:

RNIARHLAQV GDSMD (SEQ. No. 31).

Bid protein belongs to a Bcl-2 family and is responsible inter alia for activation of pro-apoptotic factor Bax. It is believed that the 16-amino acid peptide comprising the BH3 domain of Bid protein incorporated into the fusion protein of the invention will effectively induce apoptosis.

The effector peptide of SEQ. No. 32 of the above group is a peptide homologue of ribonuclease A (RNase A), presented by:

KETA AKFERQHMDS STSAASSSNY CNQMMKSRNL TKDRCKPVNT FVHESLADVQ

AVCSQKNVAC KNGQTNCYQS YSTMSITDCR ETGSSKYPNC AYKTTQANKH

IIVACEGNPY VPVHFDASV (SEQ. No. 32).

Ribonucleases are small proteins with potential antineoplastic properties, which upon binding to negatively charged cell membranes enter the cell via endocytosis, and then leak into cytosol, where they act as an enzyme to cause degradation of RNA. Starting from a concentration of 10 nM they arrest cell cycle and cause apoptosis.

The effector peptide of SEQ. No. 33, of the above described group is the cytochrome C molecule presented by:

GDVEK GKKIFIMKCS QCHTVEKGGK HKTGPNLHGL FGRKTGQAPG YSYTAANKNK

GIIWGEDTLM EYLENPKKYI PGTKMIFVGI KKKEERADLI AYLKKATNE (SEQ. No. 33).

The release of cytochrome C from mitochondria to cytoplasm is one of the main signals inducing apoptosis via so called mitochondrial path. The protein is part of apoptosome complex, which activates caspase 9.

The effector peptide of SEQ. No. 34, of the above described group is granzyme B, presented by:

IIGGHVAKPH SRPYMAYLMI WDQKSLKRCG GFLIRDDFVL TAAHCWGSSINVTLGAHNIK

EQEPTQQFIP VKRAIPHPAY NPKNFSNDIM LLQLERKAKR

TRAVQPLRLP SNKAQVKPGQ TCSVAGWGQT APLGKHSHTL QEVKMTVQED

RKCESDLRHY YDSTIELCVG DPEIKKTSFK GDSGGPLVCN KVAQGIVSYG

RNNGMPPRAC TKVSSFVHWI KKTMKRY (SEQ. No. 34).

Granzymes, also called fragmentins in the literature, are serine proteases typical for cellular granularity of Tc lymphocytes and NK cells. In human, currently 5 different granzymes are identified: A, B, H, K (tryptase) and M (metioninase). Studies have confirmed that these enzymes are the elements of the cytotoxic reaction exerted by lymphocytes against target cells. These enzymes have been shown to activate the perforin - a protein generating pores in cell membranes and thereby mediate the cytotoxic response. Furthermore, it is believed that these enzymes are directly involved in induction of apoptosis in target cells. Granzyme B activates selected procaspases into their active forms (e.g. caspase 3), and also releases via proteolysis the active form of Bid protein (a protein belonging to the Bcl-2 protein family), which initiates intracellular pathway of apoptosis by incorporation into the mitochondrial membranes and generation of pores in membranes, followed by release of apoptosis-inducing factors (cytochrome C, caspase 9, Apaf). By binding to histones Granzyme B may also participate in the relaxation of chromatin structure, which causes its relaxation and increases the access to DNA for endonucleases.

The effector peptide of SEQ. No. 35 of the above group is the fragment of Nur77 protein presented by:

FSRSLHSLL (SEQ. No. 35).

Nuclear receptor Nur77 is a very potent inducer of apoptosis. One of the mechanisms of its action is the ability to bind to Bcl-2 protein, an important anti-apoptotic factor. This interaction causes conformational changes in the structure of Bcl-2, which convert it into an inducer of apoptosis. The fragment presented above is a 9-amino acid region from the sequence of Nur77 identified as responsible for binding and conversion of Bcl-2 and induction of apoptosis in cells. (Kolluri et al, Cancer Cell 14: 285-298, 2008).

The effector peptide of SEQ. No. 36 of the above group is an 15-amino acid peptide comprising the BH3 domain of Bak protein, presented by

GQVGRQLAII GDDIN (SEQ. No. 36).

It is believed that this short peptide incorporated into the fusion protein of the invention will effectively induce apoptotic signal.

The effector peptide of SEQ. No. 37 of the above group is the BH3 domain of the protein PUMA/BBC3, presented by

EEQWAREIGA QLRRMADDLN AQYE SEQ. No. 37.

PUMA/BBC3 (p53 upregulated modulator of apoptosis/Bcl-2-binding component 3) is a member of a Bcl-2 proteins family (BH3-only subfamily). It mediates apoptosis in the manner both dependent and independent on p53. Direct interactions of PUMA/BBC3 with all known pro-survival Bcl-2 proteins cause their inactivation, mitochondrial dysfunction, and thus activation of caspases and cell death. PUMA also affects indirectly the restoration of proapoptotic activity of molecules such as Bak and Bax. The BH3 domain is responsible for binding of PUMA with pro-survival proteins.

The effector peptide of SEQ. No. 38 of the above group is a protein PUMA/BBC3, presented by

ARAR QEGSSPEPVE GLARDGPRPF PLGRLVPSAV SCGLCEPGLA AAPAAPTLLP

AAYLCAPTAP PAVTAALGGS RWPGGPRSRP RGPRPDGPQP SLSLAEQHLE

SPVPSAPGAL AGGPTQAAPG VRGEEEQWAR EIGAQLRRMA DDLNAQYERR

RQEEQQRHRP SPWRVLYNLI MGLLPLPRGH RAPEMEPN (SEQ. No. 38).

It is believed that both the protein PUMA/BBC3 and its BH3 domain when incorporated into the fusion protein of the invention will effectively induce apoptotic signals.

The effector peptide of SEQ. No. 39 of the above group is 8-amino acid fragment of the protein SMAC/Diablo, presented by

AVPIAQKP (SEQ. No. 39).

SMAC/DIABLO (Second mitochondria-derived activator of Caspase/Direct IAP Binding Protein with Low PI) is an activator of caspases released from mitochondria. Its N-terminal motif competitively binds to IAP proteins, preventing their BIR 2 and BIR 3 domains from inactivation of caspases. It is believed that this short peptide when incorporated into the fusion protein of the invention will effectively induce apoptotic signal.

The effector peptide of SEQ. No. 40 of the above group is buforin IIb peptide presented by

RAGLQFPVGR LLRRLLRRLL (SEQ. No. 40).

Buforin IIb is a peptide derived from histone H2A, which is able to independent penetration of the cell membrane and has antibacterial properties (Park et al, Biochem Biophys. Res. Commun., 244: 253-257, 1998). Studies on its utility as an anticancer agent showed that it is able to bind selectively to numerous cancer cells, penetrate the cells and accumulate in the nucleus, inducing apoptosis via the mitochondrial pathway (Lee et al, Cancer Letters, 271:47-55, 2008).

The effector peptide of SEQ. No. 41 of the above group is onconase peptide presented by

QDWLT FQKKHITNTR DVDCDNIMST NLFHCKDKNT FIYSRPEPVK AICKGIIASK

NVLTTSEFYL SDCNVTSRPC KYKLKKSTNK FCVTCENQAP VHFVGVGSC (SEQ. No. 41).

Onconase or P-30 is the protein originally derived from lysates of frog Rana pipiens oocytes. It is a single-stranded protein with the mass of 12 kDa, structural homolog of RNase A. The research on this protein showed that it has a remarkable cytotoxic activity against tumour cells (Y Wu, SM Mikulski, W Ardelt, SM Rybak and RJ Youle, The Journal of Biological Chemistry 268, 10686-10693). The research on the mechanism of action of onconase showed that upon the internalization process it enters into the cell, where carries out the degradation process of 28S and 18S ribosomal rRNA, which leads to inhibition of protein synthesis and cell death.

The effector peptide of SEQ. No. 42 of the above group is the 20-amino acid N-terminal fragment of p14ARF protein, which is the inhibitor of pro-survival Mdm2 protein, presented by

VRRFLVTLRI RRACGPPRV (SEQ. No. 42).

P14ARF is the protein that regulates the activity of Mdm2 protein, which binds to the tumour suppressor p53 and is responsible for its degradation and thereby possibility of survival of transformed cells. P14ARF protein by binding to Mdm2 prevents its interaction with p53. It is reported that a short peptide derived from p14ARF is sufficient to block the interaction between Mdm2 and

p53 and prevent the degradation of the latter (Midgley et al, Oncogene 19: 2312-2323, 2000).

The effector peptide of SEQ. No. 43 of the above group is the 11-amino acid peptide binding to Mdm2 presented by

PRFMDTWEGL N (SEQ. No. 43).

The above peptide shows sequence homology to the sequence of p53 and significant efficiency of inhibition of Mdm2-p53 interactions (Böttger et al, Oncogene 13:2141-2147, 1996), thereby preventing the degradation of p53.

The effector peptide of SEQ. No. 44 of the above group is the 17-amino acid fragment of the lunasin peptide presented by

CEKHIMEKIQ GRGDDDD (SEQ. No. 44).

Lunasin is a 43-amino acid peptide derived from soybeans (Glycine max) with proven anti-carcinogenic potential. The general mechanism of action of this molecule consists in inhibition of histone acetylation. It is known that molecules that possess deacetylase activity act also as co-suppressors of transcription process (Leong et al, Cancer Lett, 18: 42 - 48, 2007).

The effector peptide of SEQ. No. 45 of the above group is the BH3 domain of Bik protein presented by

LALRLAC IGDEMDVS (SEQ. No. 45).

Bik protein interacts with cellular and viral factors initiating the survival signals (e.g. Bcl-2), thereby stimulating apoptosis. Like many other pro-apoptotic proteins, it contains a BH3 domain necessary for interaction with Bcl-2. A peptide derived from this protein comprising the BH3 domain may initiate apoptosis by activating other pro-apoptotic proteins or by inhibiting anti-apoptotic proteins (Del Gaizo Moore, V, et al, Blond, 111: 2300-2309, 2008).

The effector peptide of SEQ. No. 46 of the above group is the synthetic peptide - a proteasome inhibitor presented by

AGAGGGAGG AGAGGGAGGA G (SEQ. No. 46).

This peptide consists of a series of repetitions of Gly and Ala residues, and is proteasome inhibitor capable of potentation of TRAIL-induced apoptosis by induction of overexpression of the TRAIL receptor DR5.

The effector peptide of SEQ. No. 47 of the above group is the domain of the C-terminal fragment of proteasome S5a presented by

MTISQQEFG RTGLPDLSSM TEEEQIAYAM QMSLQGAEFG QAESADIDAS SAMDTSEPAK

EEDDYDVMQD PEFLQSVLEN LPGVDPNNEA IRNAMGSLAS QATKDGKKDK KEEDK (SEQ. No. 47).

This domain from the proteasome S5a fragment contains UIMs motifs that directly participate in ubiquitin binding and thus have the ability to induce apoptosis.

The effector peptide of SEQ. No. 151 of the above group is the azurin derived peptide.

Azurin, a copper-containing redox protein, released by the pathogenic bacterium Pseudomonas aeruginosa, is highly cytotoxic to many cancer cell lines. It enters into the cytosol and travels to the nucleus. It's activity strictly depends on the presence of active form of p53 in cancer cells. Azurin has been shown to bind p53 and post-translationaly increase the p53 and Bax level. This apparent antagonistic action with respect to the Mdm2-p53 functional interaction suggests that binding of Azurin to p53 might interfere with the Mdm2-p53 association and thus prevent degradation of p53. Upon binding, it triggers the release of mitochondrial cytochrome C into cytosol. This process activates the caspase cascade (including caspase-9 and caspase-7), thereby initiating the apoptotic process (Punj V, et al Oncogene. 2004 Mar 25;23(13):2367-78, Funari G et al. J Mol Recognit. 2010 Jul Aug;23(4):343-51). Detailed analysis of activity of peptides derived from azurin sequence revealed the region of 28 amino acids responsible for efficient cell penetration and triggering apoptosis (Yamada i wsp., Cell Microbiol, 7:1418-1431, 2005).

The effector peptide of SEQ. No. 152 of the above group is the full length azurin peptide.

The effector peptide of SEQ. No. 153 of the above group is the peptide designed from aPP protein and BH3 domain of Bax protein.

Chimeras of aPP protein and redesigned pro-apoptotic Bak protein were reported in EP1309680 as highly potent and specific ligands for human Bcl-2 and Bcl-X. (See also Chin JW, Schepartz A. Design and evolution of a miniature Bcl-2 binding protein Angew Chem Int Ed Engl. 2001 Oct 15;40(20):3806-3809).

The effector peptide of SEQ. No. 154 of the above group is an another peptide designed from aPP protein and BH3 domain of Bax protein.

The effector peptide of SEQ. No. 155 of the above described group is the Reticulon RTN1-C derived peptide.

RTN1-C protein is a membrane protein localized in the ER and expressed in the nervous system, and its biological role is not completely clarified. The C-terminal region of RTN1-C, corresponding to the fragment from residues 186 to 208 is able to bind the nucleic acids and to interact with histone deacetylase (HDAC) enzymes decresing their activity.

The effector peptide of SEQ. No. 156 of the above group is the full length human Reticulon 3 (isoform a). Reticulons (RTNs) form a group of integral membrane proteins that have no homology to other known apoptosis-related domains. Reticulon 3 isoform a is overexpresed in tumor cell lines, turning them to be sensitive to TRAIL-mediated apoptosis.

The effector peptide of SEQ. No. 157 of the above group is the modified constutively active caspase-3 (single chain) (Srinivasula SM, Ahmad M, MacFarlane M, Luo Z, Huang Z, Fernandes-Alnemri T, Alnemri ES. Generation of constitutively active recombinant caspases-3 and -6 by rearrangement of their subunits. J Biol Chem. 1998 Apr 24;273(17):10107-11).

The effector peptide of SEQ. No. 158 of the above group is the SAC domain from Par-4 protein (prostate apoptosis response protein par-4).

Par-4 is a tumor suppressor protein with a pro-apoptotic function. The cancer-specific pro-apoptotic action of Par-4 resides in its centrally located SAC domain. The function of the molecule is achieved by two distinct means: activation of molecular components of the cell-death machinery (translocation of Fas and FasL to the plasma membrane), and inhibition of pro-survival factor (NF-κB pathway). (Zhao Y, Rangnekar VM. Apoptosis and

tumor resistance conferred by Par-4. Cancer Biol Ther. 2008 Dec;7(12):1867-74. Epub 2008 Dec 8. Review).

The effector peptide of SEQ. No. 159 of the above group is the Noxa protein. Noxa encodes a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins; this member contains the BH3 region but not other BH domains. Noxa is a mediator of p53-dependent apoptosis and undergoes BH3 motif-dependent localization to mitochondria and interacts with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9.

The effector peptide of SEQ. No. 160, of the above described group is the 10 AA (KLLNLISKLF) fragment of Noxa protein required for mitochondrial location (MTD-mitochondrial targetting domain or CKP - Cell Killing Peptide). It was described in WO2006/001582 and in Young-Woo Seo et al. in The Journal of Biological Chemistry Vol. 278, No. 48, Issue of November 28, pp. 48292-48299, 2003.

The effector peptide of SEQ. No. 161 of the above group is the short hybrid peptide Antp-TPR described in WO2010055929. Antp-TPR is an engineered hybrid peptide targeting Hsp90, which has selective cytotoxic activity towards cancer cells due to inhibition of the interaction of Hsp90 with the TPR2A domain of Hop.

The effector peptide of SEQ. No. 162, of the above described group is the peptide inhibitor of the SH2 domain of Stat3 protein.

SH2 domain of Stat proteins is responsible for the series of events that lead to promoting cell growth and differentiation via normal STAT signalling in response to growth factors and cytokines.

The effector peptide of SEQ. No. 163 of the above group is the peptide GQVGRQLAIIGDDINR derived from BH3 domain of Bak protein (Bcl-2 family) (Castelli M, Reiners JJ, Kessel D. A mechanism for the proapoptotic activity of ursodeoxycholic acid: effects on Bcl-2 conformation. Cell Death Differ. 2004 Aug;11(8):906-14). The Bak protein is a pro-apoptotic member of the Bcl-2 family which is involved in apoptosis initiation.

The effector peptide of SEQ. No. 164 of the above group is the peptide KNLWAAQRYGRELRRMSDEFEGSFKGL derived from BH3 domain of Bad protein

(Bcl-2 family) (Wang JL, Zhang ZJ, Choksi S, Shan S, Lu Z, Croce CM, Alnemri ES, Korngold R, Huang Z. Cell permeable Bcl-2 binding peptides: a chemical approach to apoptosis induction in tumor cells. Cancer Res. 2000 Mar 15;60(6):1498-502).

The effector peptide of SEQ. No. 165 of the above group is the peptide ATAP from Bfl1 protein.

ATAP (amphipathic tail-anchoring peptide) (residues 147-175 from Bfl1, a bifunctional Bcl2 family protein), targets specifically to mitochondria and induces caspase-dependent apoptosis that does not require Bax or Bak.

The effector peptide of SEQ. No. 166 of the above group is another ATAP peptide from Bfl1 protein. The ATAP protein is fused to MTS domain from HCCS1 (Ko JK, Choi KH, Pan Z, Lin P, Weisleder N, Kim CW, Ma J. The tail-anchoring domain of Bfl1 and HCCS1 targets mitochondrial membrane permeability to induce apoptosis. J Cell Sci. 2007 Aug 15;120(Pt 16):2912-23. Epub 2007 Jul 31).

As described herein above, the first variant of the pro-apoptotic effector peptide of domain (b) may be a peptide exerting its apoptotic activity via intrinsic apoptosis pathway (intracellularly), that induces apoptosis directly by activating signalling cascade components of mitochondrial pathway of apoptosis, or by direct induction of mitochondrial apoptosis in cells.

In one embodiment of the first variant, one group of pro-apoptotic effector peptides of domain (b) exerting its activity via intrinsic pathway may be peptides that inhibit and/or modulate intracellular anti-apoptotic or pro-survival factors, such as anti-apoptotic proteins Bcl-2 and Bcl-XL, upon binding thereof.

Exemplary effector peptides of the above group are peptides represented by SEQ. No. 30, present in the fusion proteins of Examples 1, SEQ. No. 37 present in the fusion proteins of Examples 11 and 47, SEQ. No. 45 incorporated in the fusion protein of Example 21, SEQ. No. 158 present in the fusion proteins of Examples 42 and 43, and SEQ. No. 159 incorporated in the fusion protein of Example 44.

In another embodiment of this first variant, a group of pro-apoptotic effector peptides of domain (b) exerting its activity via intrinsic pathway may be peptides exerting direct destructive effect inside the cell to arrest the cell cycle.

Said direct destructive effect inside the cell in a mitochondrial intrinsic pathway may be initiated by the effector peptide on different levels of the caspase cascade leading to cell death.

Examples of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway are degradation of nucleic acids, particularly whole cellular RNA or DNA and induction of degradative nucleases. Such an effect may be exerted for example by ribonucleases, such as ribonucleases of superfamily of pancreatic RNAse A, including human pancreatic RNAse, human angiogenin (ribonuclease 5, hAng), human eosinophil-derived neurotoxin (EDN) and bovine ribonuclease, as well as their homologs and variants. Examples of RNAse homologs are onconase, ribonucleases isolated from Rana catesbiana and Rana japonica.

Exemplary effector peptides of the above group acting by degradation of nucleic acids are peptides represented by SEQ. No. 32, present in the fusion proteins of Examples 3, 4 and 27, SEQ. No. 41, present in the fusion proteins of Examples 16, 17 and 46, and SEQ. No. 157, present in the fusion protein of Example 41.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is caspase activation. Such an effect may be exerted for example by cytochrome c (SEQ. No. 33), present in the fusion proteins of Examples 5 and 6, granzyme B (SEQ. No. 34), present in the fusion proteins of Examples 7 and 8, or peptide derived form protein Smac/DIABLO (SEQ. No. 39), present in the fusion proteins of Examples 14, 21, 33, 34 and 35.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is proteasome inhibition, due to influence of the stabilisation of proapoptotic proteins on restoration of p53 functions.

Exemplary effector peptides of the above group acting by proteasome inhibition are peptides represented by SEQ. No. 46 incorporated in the fusion

protein of Example 22, and SEQ. No. 47 incorporated in the fusion protein of Example 23.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is modulation of histone proteins due enhancement of the influence of expression of proapoptotic proteins on restoration of p53 functions.

Exemplary effector peptides of the above group acting by modulation of histone proteins are buforin IIb represented by SEQ. No. 40 incorporated in the fusion protein of Example 15 and lunasin represented by SEQ. No. 44 incorporated in the fusion protein of Example 20.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is restoration of p-53 functions, such as by inhibition of its degradation. Prevention of p-53 degradation may be achieved by inhibition of the negative regulator of p-53, such as murine double minute 2 (MDM2) to disrupt its negative regulation. This may be achieved by MDM2 binding peptides, which compete with MDM2 for binding to p-53, such as Azurin, a copper-containing redox protein, a cycle cell regulator p14ARF, or SuperTIP (ThioredoxinInsert Protein, mdm-2-binding peptide within the active site loop of the bacterial thioredoxin protein), or their fragments.

Exemplary effector peptides of the above group acting by restoration of p-53 functions are peptides represented by SEQ. No. 42 incorporated in the fusion protein of Example 18, SEQ. No. 43 incorporated in the fusion protein of Example 19, SEQ. No. 151 present in the fusion proteins of Examples 29, 30 and 31, and SEQ. No. 152 incorporated in the fusion protein of Example 32.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is influencing, i.e. activation, inhibition or modulation of Bcl-2 proteins family, such as proteins Bax, Bak, Bok, Bid, Bim, Bad, Bmf, Hrk, Noxa, Puma, Bik, BNIP3 and Spike, more particularly BH3 only - proteins family, including Bid, Bim, Bad, Bmf, Hrk, Noxa, Puma, Bik, BNIP3 and Spike. In particular, fragments of BH3 domains of Bcl-2 family members will be advantageous effector peptides. Other group of effector peptides are fragments of the family of nuclear receptors RXR (Retinoid X Receptor), such as for example nuclear receptor Nur77.

Exemplary effector peptides of the above group acting by influencing Bcl-2 proteins family are peptides represented by SEQ. No. 30 incorporated in the fusion protein of Example 1, SEQ. No. 31 present in the fusion proteins of Examples 2, 4 and 8, SEQ. No. 32 incorporated in the fusion protein of Example 3, SEQ. No. 35 incorporated in the fusion protein of Example 9, SEQ. No. 36 incorporated in the fusion protein of Example 10, SEQ. No. 37 present in the fusion proteins of Examples 11 and 47, SEQ. No. 38 present in the fusion proteins of Examples 12 and 13, SEQ. No. 159 incorporated in the fusion protein of Example 44, SEQ. No. 160 incorporated in the fusion protein of Example 45, SEQ. No. 163 incorporated in the fusion protein of Example 51, SEQ. No. 164 present in the fusion proteins of Examples 52 and 53, SEQ. No. 165 incorporated in the fusion protein of Example 54, and SEQ. No. 166 incorporated in the fusion protein of Example 55.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is converging apoptotic signal induced by TRAIL binding to TRAIL receptors, particularly by caspase activation.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is promoting apoptosome formation.

Exemplary effector peptides of the above group acting by promoting apoptosome formation are peptides represented by SEQ. No. 30 incorporated in the fusion protein of Example 1, SEQ. No. 31 incorporated in the fusion protein of Example 2, SEQ. No. 33 present in the fusion proteins of Examples 5 and 6, SEQ. No. 35 incorporated in the fusion protein of Example 9, SEQ. No. 36 incorporated in the fusion protein of Example 10, SEQ. No. 37 incorporated in the fusion protein of Example 47, SEQ. No. 39 present in the fusion proteins of Examples 33, 34 and 35, SEQ. No. 40 incorporated in the fusion protein of Example 14, SEQ. No. 45 incorporated in the fusion protein of Example 21, SEQ. No. 153 present in the fusion proteins of Examples 36 and 37, SEQ. No. 154 incorporated in the fusion protein of Example 38, SEQ. No. 157 incorporated in the fusion protein of Example 41, SEQ. No. 158 present in the fusion proteins of Examples 42 and 43, SEQ. No. 159 incorporated in the fusion protein of Example 44, SEQ. No. 160 incorporated in the fusion protein of Example 45, SEQ. No. 163 incorporated in the fusion protein of Example 51, and SEQ. No. 164 present in the fusion proteins of Examples 52 and 53.

Another example of said direct destructive effect of the effector peptide in a mitochondrial intrinsic pathway is promoting mitochondrial outer membrane (MOMP) permeabilization, due to which proteins released by mitochondrium can act on caspase activation level.

Exemplary effector peptides of the above group acting by promoting MOMP permeabilization are peptides represented by SEQ. No. 30 incorporated in the fusion protein of Example 1, SEQ. No. 31 present in the fusion proteins of Examples 2 and 48, SEQ. No. 33 present in the fusion proteins of Examples 5 and 6, SEQ. No. 39 present in the fusion proteins of Examples 14, 33, 34 and 35, SEQ. No. 40 incorporated in the fusion protein of Example 15, SEQ. No. 41 incorporated in the fusion protein of Example 46, and SEQ. No. 45 incorporated in the fusion protein of Example 21.

As described herein above, the second variant of the pro-apoptotic effector peptide of domain (b) of the invention is the group of pro-apoptotic effector peptides acting via extrinsic pathway (extracellularly), which for their effect require binding to receptors present on the surface of the cancer cell.

The following TNF-ligands (TNF - tumour necrosis factor) or TNF-analogs as extracellularly acting peptides were used as the comparative effector peptides:

- VANPQAEGQL decapeptide (SEQ. No. 48);
- LANGVE hexapeptide (SEQ. No. 49), or
- Septapeptide CPSEGLC (SEQ. No. 50).

Decapeptide represented by SEQ. No. 48 has been described as analog/agonist of TNF in JP 60,226,816.

Hexapeptide represented by SEQ. No. 49 derives from TNF and has been described in DE 3,841,768.

Septapeptide represented by SEQ. No. 50 is the five-amino acid peptide, which is a part of the TNF cytokine derived from the surface of the interaction of this cytokine with its cellular receptors: TNFR55 and TNFR75, which is flanked at the C-terminus and the N-terminus by two cysteine residues. Cysteine residues stabilize peptide cyclization via formation of a

sulphide bridge between the amino acids. The aim of cyclization is stabilization of the peptide and improvement of its activity.

Upon binding to TRAIL receptors present on the surface of cancer cells, the fusion protein will exert a double effect. Domain (a), that is a functional fragment of TRAIL, will exert its known agonistic activity - i.e. binding to death receptors on the cell surface and activation of the extrinsic pathway of apoptosis. After internalization via endocytosis of the fusion protein comprising pro-apoptotic peptide acting intracellularly, the domain (b) will be able to potentially exert its action intracellularly parallel to the activity of TRAIL domain. In this way, anti-cancer activity of TRAIL can be potentiated by activation of other elements and mechanisms of apoptosis.

The comparative fusion protein incorporating pro-apoptotic peptide acting extracellularly should potentially additionally initiate apoptosis pathway by binding to and activating pro-apoptotic receptors other than TRAIL receptors.

In one of the embodiments of the invention, domains (a) and (b) of the fusion protein can be linked directly with each other.

In another embodiment, domain (a) and domain (b) are linked by a domain (c) comprising the sequence of a cleavage site recognized by proteases present in the cell environment, especially in the tumour cell environment.

A protease cleavage site can be selected from:

- a sequence recognized by the metalloprotease MMP, in particular sequences PLGLAG (SEQ. No. 51), PLGIAGE (SEQ. No. 171) or PLGLAGQ (SEQ. No. 173),

- a sequence recognized by urokinase uPA, in particular RVVR sequence (SEQ. No. 52), and

- a sequence recognized by furin, in particular sequence RKKR (SEQ. No. 53), or sequence RKKRVKR (SEQ. No. 172),

and their combinations.

In particular, the protease cleavage site is a combination of the sequence recognized by the metalloprotease MMP and a sequence recognized by urokinase uPA, located next to each other in any order.

In one embodiment, the domain (c) is a combination of MMP/uPA SEQ. No 51/Sekw. No. 52, that is the sequence PLGLAGRVVR, or a combination of uPA/MMP SEQ. No 52/SEQ. No. 51, that is the sequence RVVRPLGLAG.

Proteases metalloprotease MMP, urokinase and/or furin are overexpressed in the tumour environment. The presence of the sequence recognized by the protease enables the cleavage of the domain (a) from the domain (b) upon internalization of the construct, i.e. the release of the functional domain (b) and thus its activation.

The presence of the protease cleavage site, by allowing quick release of the effector peptide, increases the chances of transporting the peptide to the place of its action, before random degradation of the fusion protein by proteases present in the cell occurs.

Additionally, to the domain (b) of the effector peptide of the fusion protein of the invention may be attached a transporting domain (d), selected from the group consisting of:

(d1) a sequence directing to the endoplasmic reticulum,

(d2) a polyarginine sequence transporting through the cell membrane, comprised of 6, 7, 8 or 9 Arg residues,

(d3) a translocation domain of Pseudomonas aeruginosa (SEQ. No. 54),

(d4) a membrane transporting domain,

(d5) a nuclear localization domain, and

(d6) a mitochondrial targeting domain,

and combinations thereof.

The combination of domains (d1) (d2) and (d3) may comprise, in particular the combination of (d1)/(d2), (d1)/(d3) or (d1)/(d2)/(d3).

The combination of domains (d1), (d2), (d3), (d4), and (d5) may comprise, in particular also the combination of (d1)/(d2), (d1)/(d3), (d1)/(d4), (d1)/(d5) and (d1)/(d2)/(d3), (d3)/(d5), (d2)/(d5), (d1)/(d3)/(d5), (d2)/(d3)/(d6).

Furthermore, the combination of domains (d1), (d2), (d3), (d4), and (d5) may include domains located next to each other and connected to one end of the domain (b) and/or domains linked to different ends of the domain (b).

It should be understood that in the case when the fusion protein has both the transporting domain (d) attached to the domain (b) and the domain (c) of the cleavage site between the domains (a) and (b), then the domain (c) is located in such a manner that after cleavage of the construct the transporting domain (d) remains attached to the domain (b). In other words, if the fusion protein contains both the transporting domain (d) and the cleavage site domain (c), then the domain (d) is located between the domain (b) and the domain (c), or is located at the end of the domain (b) opposite to the place of attachment of domain (d). The invention does not comprise such a variant in which the domain (d) is located between the domain (c) a domain (a), that is the case when after cleavage of the construct the transporting domain remains attached to the TRAIL domain.

The transporting sequence may be attached at the N-terminus or at the C-terminus of the domain (b). In some embodiments, the transporting sequence may be also terminal part of the whole construct, such as C-terminal part or N-terminal part, depending on the manner of attachment of domains (a) and (b).

Translocation domain of *Pseudomonas aeruginosa* is capable of translocation through the lysosomal membrane into the cytoplasm and can be used to introduce the effector peptide to the tumour cell compartments. The translocation domain sequence of *Pseudomonas aeruginosa* is well known and is represented by:

PEGGSLA ALTAHQACHL PLETFTRHRQ PRGWEQLEQC GYPVQRLVAL YLAARLSWNQ

VDQVIANALA SPGSGGDLGE AIRESPEQAR LALTLAAAES ERFVRQGTGN

DEAGAANGPA D (SEQ. No. 54)

The sequence (d1) directing to endoplasmic reticulum may be any signal sequence directing to endoplasmic reticulum known in the art, such as for example, but not limited to, KDEL, HDEL, RDEL, DDEL, ADEL, SDEL, KEDL. Sequence (d1) is preferably selected from the sequences KDEL (SEQ. No. 55) and KEDL (SEQ. No. 56).

Preferably, the directing sequence (d1) is located at the C-terminus of the fusion protein of the invention and forms C-terminal part thereof.

The membrane transporting domain (d4) may be any signal sequence transporting through the plasma membrane known in the art, such as for example and not limited to, KPRRPY or K PRRPYR.

The nuclear localization sequence (d5) may be any signal sequence directing into nucleus known in the art, such as for example and not limited to, EEEAAGRKRKKRT (SEQ. No. 168), FFFAAGRKRKKRT, NNNAAGRKRKKRT, YYYAAGRKRKKRT, AAKKK, or GR KRKKRT.

The mitochondrial targeting domain (d6) may be any signal sequence directing to mitochondrium known in the art, such as for example and not limited to RVSFCRPGWSAMARSRLTATSVSQVQENGFVK (SEQ. No. 166), fragment MLATRVFSLVGKRAISTSVCVR of human cytochrome oxidase subunit IV (hCOXIV1), or the ornithine transcarbamylase leader peptide.

Apart from the main functional elements of the fusion protein, transporting domains and the cleavage site domains, the fusion proteins of the invention may contain domain (e), i.e. a polycysteine motif facilitating trimer stabilisation, as, for example, and not limited to, CAACAAAC sequence (SEQ. No. 177) or CAAECAAAC (SEQ. No. 178).

Furthermore, the polycysteine domain (e) may by connected to one end of the domain (b) and/or linked to different ends of the domain (b).

It should be understood that in the case when the fusion protein has both the polycysteine domain (e) attached to the domain (b) and the domain (c) of the cleavage site between the domains (a) and (b), then the domain (c) is located in such a way that after cleavage of the construct the polycysteine domain (e) remains attached to the domain (a). In other words, if the fusion protein contains both polycysteine domain (e) and cleavage site domain (c), then domain (e) is located between domain (a) and domain (c), or is placed at the end of the domain (a) opposite to the place of attachment of domain (d). The invention does not comprise such a variant in which domain (e) would be located between domain (c) and domain (b), that is the case when after cleavage of the construct the polycysteine domain would remain attached to the effector peptide domain.

Apart from the main functional elements of the fusion protein, transporting domains and the cleavage site domain(s), the fusion proteins of the invention

may contain a neutral sequence/sequences of a flexible steric linker (spacer) comprised of alanine, glycine, glutamine, cysteine, histidine and serine residues. Such linkers/spacers are well known and described in the literature. Their incorporation into the sequence of the fusion protein is intended to provide the correct folding of proteins produced by the process of its overexpression in the host cells.

In particular, the flexible steric linker may be selected from the group consisting of GGSG (SEQ. No. 57), GGGS (SEQ. No. 58), GGGGS (SEQ. No. 59), GGSGG (SEQ. No. 60), GGGSGG (SEQ. No. 61), GGGSGGG (SEQ. No. 62), GGGSGGGS (SEQ. No. 63), GGGSGGGGS (SEQ. No. 64), ASGG (SEQ. No. 65), GGGSASGG (SEQ. No. 66) SGCGS (SEQ. No. 169), GGGGSGGGG (SEQ. No. 180),GGSHG (SEQ. No. 182), SGGCGGS(SEQ. No. 183) and AACAA (SEQ. No. 184).

In one of the embodiments, between domain (a) and domain (b) there is additionally

(f) domain of the sequence suitable for attachment to the fusion protein of the invention of a PEG molecule (PEG linker).

Such a linker can be a known sequence AlaSerGlyCysGlyProGlu (ASGCGPE in a one-letter convention), designated in the attached Sequence Listing as the SEQ. No. 170. PEG linker can be also chosen from between AlaAlaCysAlaAla (AACAA), SerGlyGlyCysGlyGlySer (SGGCGGS) i (SGCGS), designated in the attached Sequence Listing as, respectively, SEQ. No. 178, SEQ. No. 177 and SEQ. No. 179.

In another embodiment domains (a) (b) (c) (d) (e) and (f) may be additionally separated by up to three amino acids residues, formed of amino acid residues, particularly selected from the group consisting of Glycine and Glutamine.

Furthermore, in some embodiments the fusion protein may contain as a C-terminal part of the whole construct a non-functional fragment of hTRAIL, such as the sequence hTRAIL95-121, preceded by the sequence allowing its cleavage from the construct, advantageously the protease cleavage site, preferably the sequence recognized by thrombin. Incorporation of such small non-functional fragment of hTRAIL confers greater hydrophility to the whole construct, thus improving solubility of the protein during the expression

process. After the purification steps hTRAIL95-121 will be cleaved by thrombin. In such a case, hTRAIL95-121 will not be present in the fusion protein used for the preparation of the pharmaceutical composition.

Any sequence recognized by thrombin known in the art may be used, in particular sequence LVPRGS (SEQ. No. 174).

Such additional hTRAIL95-121 sequence is especially advantageous in the case of lipophilic effector peptides, and when domain (a) begins with the aminoacid 114 and higher in the sequence of the whole TRAIL.

Particular embodiments of the fusion protein of the invention are fusion proteins comprising an intracellularly acting proapoptotic peptide, selected from the group consisting of the proteins represented by:

SEQ. No. 1, SEQ. No. 2, SEQ. No. 3, SEQ. No. 4, SEQ., No. 5, SEQ. No. 6, SEQ. No. 7, SEQ. No. 8, SEQ. No. 9, SEQ. No. 10, SEQ. No. 11, SEQ. No. 12, SEQ. No. 13, SEQ. No. 14, SEQ. No. 15, SEQ. No. 16, SEQ. No. 17, SEQ. No. 18, SEQ. No. 19, SEQ. No. 20, SEQ. No. 21, SEQ. No. 22, SEQ. No 23, SEQ. No. 93, SEQ. No. 94, SEQ. No. 95, SEQ. No. 96, SEQ., No. 97, SEQ. No. 98, SEQ. No. 99, SEQ. No. 100, SEQ. No. 101, SEQ. No. 102, SEQ. No. 103, SEQ. No. 104, SEQ. No. 105, SEQ. No. 106, SEQ. No. 107, SEQ. No. 108, SEQ. No. 109, SEQ. No. 110, SEQ. No. 111, SEQ. No. 112, SEQ. No. 113, SEQ. No. 114, SEQ. No 115, SEQ. No. 116, SEQ. No. 117, SEQ. No. 118, SEQ. No. 119, SEQ. No. 120 and SEQ. No. 121.

Other specific embodiments of the fusion protein of the invention are fusion proteins comprising an extracellularly acting pro-apoptotic peptide selected from the group consisting of proteins represented by SEQ. No. 24, SEQ. No. 25 and SEQ. No. 26.

A detailed description of the structure of representative fusion proteins mentioned above are shown in Figures 1 to 5 and 9 to 13, and in the Examples presented herein below.

In accordance with the present invention, by the fusion protein it is meant a single protein molecule containing two or more proteins or fragments thereof, covalently linked via peptide bond within their respective peptide chains, without additional chemical linkers.

The fusion protein can also be alternatively described as a protein construct or a chimeric protein. According to the present invention, the terms "construct" or "chimeric protein", if used, should be understood as referring to the fusion protein as defined above.

For a person skilled in the art it will be apparent that the fusion protein thus defined can be synthesized by known methods of chemical synthesis of peptides and proteins.

The fusion protein can be synthesized by methods of chemical peptide synthesis, especially using the techniques of peptide synthesis in solid phase using suitable resins as carriers. Such techniques are conventional and known in the art, and described inter alia in the monographs, such as for example Bodanszky and Bodanszky, The Practice of Peptide Synthesis, 1984, Springer-Verlag, New York, Stewart et al., Solid Phase Peptide Synthesis, 2nd Edition, 1984, Pierce Chemical Company.

The fusion protein can be synthesized by the methods of chemical synthesis of peptides as a continuous protein. Alternatively, the individual fragments (domains) of protein may be synthesized separately and then combined together in one continuous peptide via a peptide bond, by condensation of the amino terminus of one peptide fragment from the carboxyl terminus of the second peptide. Such techniques are conventional and well known.

For verification of the structure of the resulting peptide known methods of the analysis of amino acid composition of peptides may be used, such as high resolution mass spectrometry technique to determine the molecular weight of the peptide. To confirm the peptide sequence protein sequencers can also be used, which sequentially degrade the peptide and identify the sequence of amino acids.

Preferably, however, the fusion protein of the invention is a recombinant protein, generated by methods of gene expression of a polynucleotide sequence encoding the fusion protein in host cells.

A further aspect of the invention is the polynucleotide sequence, particularly DNA sequence encoding a fusion protein as defined above.

Preferably, the polynucleotide sequence, particularly DNA, according to the invention, encoding the fusion protein as defined above, is a sequence optimized for expression in *E. coli*.

Another aspect of the invention is also an expression vector containing the polynucleotide sequence, particularly DNA sequence of the invention as defined above.

Another aspect of the invention is also a host cell comprising an expression vector as defined above.

A preferred host cell for expression of fusion proteins of the invention is an *E. coli* cell.

Methods for generation of recombinant proteins, including fusion proteins, are well known. In brief, this technique consists in generation of polynucleotide molecule, for example DNA molecule encoding the amino acid sequence of the target protein and directing the expression of the target protein in the host. Then, the target protein encoding polynucleotide molecule is incorporated into an appropriate expression vector, which ensures an efficient expression of the polypeptide. Recombinant expression vector is then introduced into host cells for transfection/transformation, and as a result a transformed host cell is produced. This is followed by a culture of transformed cells to overexpress the target protein, purification of obtained proteins, and optionally cutting off by cleavage the tag sequences used for expression or purification of the protein.

Suitable techniques of expression and purification are described, for example in the monograph Goeddel, Gene Expression Technology, Methods in Enzymology 185, Academic Press, San Diego, CA (1990), and A. Staron et al., Advances Mikrobiol., 2008, 47, 2, 1983-1995.

As expression vectors for the introduction and replication of DNA sequences in host cells can be used cosmids, plasmids or modified viruses. Typically plasmids are used as expression vectors. Suitable plasmids are well known and commercially available.

Expression vector of the invention comprises a polynucleotide molecule encoding the fusion protein of the invention and the necessary regulatory

sequences for transcription and translation of the coding sequence incorporated into a suitable host cell. Selection of regulatory sequences is dependent on the type of host cells and can be easily carried out by a person skilled in the art. Examples of such regulatory sequences are transcriptional promoter and enhancer or RNA polymerase binding sequence, ribosome binding sequence, containing the transcription initiation signal, inserted before the coding sequence, and transcription terminator sequence, inserted after the coding sequence. Moreover, depending on the host cell and the vector used, other sequences may be introduced into the expression vector, such as the origin of replication, additional DNA restriction sites, enhancers, and sequences allowing induction of transcription.

The expression vector will also comprise a marker gene sequence, which confers defined phenotype to the transformed cell and enables specific selection of transformed cells. Furthermore, the vector may also contain a second marker sequence which allows to distinguish cells transformed with recombinant plasmid containing inserted coding sequence of the target protein from those which have taken up the plasmid without insert. Most often, typical antibiotic resistance markers are used, however, any other reporter genes known in the field may be used, whose presence in a cell (in vivo) can be easily determined using autoradiography techniques, spectrophotometry or bio- and chemi-luminescence. For example, depending on the host cell, reporter genes such as ß-galactosidase, ß-gluckuronidase, luciferase, chloramphenicol acetyltransferase or green fluorescent protein may be used.

Furthermore, the expression vector may contain signal sequence, transporting proteins to the appropriate cellular compartment, e.g. periplasma, where folding is facilitated. Additionally a sequence encoding a label/tag, such as HisTag attached to the N-terminus or GST attached to the C-terminus, may be present, which facilitates subsequent purification of the protein produced using the principle of affinity, via affinity chromatography on a nickel column. Additional sequences that protect the protein against proteolytic degradation in the host cells, as well as sequences that increase its solubility may also be present.

Auxiliary element attached to the sequence of the target protein may block its activity, or be detrimental for another reason, such as for example due to toxicity. Such element must be removed, which may be accomplished by enzymatic or chemical cleavage.

In particular, a six-histidine tag HisTag or other markers of this type attached to allow protein purification by affinity chromatography should be removed, because of its described effect on the liver toxicity of soluble TRAIL protein.

Heterologous expression systems based on various well-known host cells may be used, including prokaryotic cells: bacterial, such as *Escherichia coli* or *Bacillus subtilis*, yeasts such as *Saccharomyces cervisiae* or *Pichia pastoris*, and eukaryotic cell lines (insect, mammalian, plant).

Preferably, due to the ease of culturing and genetic manipulation, and a large amount of obtained product, the *E. coli* expression system is used. Accordingly, the polynucleotide sequence containing the target sequence encoding the fusion protein of the invention will be optimized for expression in *E. coli*, i.e. it will contain in the coding sequence codons optimal for expression in E.coli, selected from the possible sequence variants known in the state of art. Furthermore, the expression vector will contain the above-described elements suitable for *E. coli* attached to the coding sequence.

Accordingly, in a preferred embodiment of the invention a polynucleotide sequence comprising a sequence encoding a fusion protein of the invention, optimized for expression in *E. coli* is selected from the group of polynucleotide sequences consisting of:

SEQ. No. 67, SEQ. No. 68, SEQ. No. 69, SEQ. No. 70, SEQ. No. 71, SEQ. No. 72, SEQ. No. 73, SEQ. No. 74, SEQ. No. 75, SEQ. No. 76, SEQ. No. 77, SEQ. No. 78, SEQ. No. 79, SEQ. No. 80, SEQ. No. 81, SEQ. No. 82, SEQ. No. 83, SEQ. No. 84, SEQ. No. 85, SEQ. No. 86, SEQ. No. 87, SEQ. No. 88), SEQ. No. 89, SEQ. No. 90, SEQ. No. 91, SEQ. No. 92, SEQ. No. 122, SEQ. No. 123, SEQ. No. 124, SEQ. No. 125, SEQ. No. 126, SEQ. No. 127, SEQ. No. 128, SEQ. No. 129, SEQ. No. 130, SEQ. No. 131, SEQ. No. 132, SEQ. No. 133, SEQ. No. 134, SEQ. No. 135, SEQ. No. 136, SEQ. No. 137, SEQ. No. 138, SEQ. No. 139, SEQ. No. 140, SEQ. No. 141, SEQ. No. 142, SEQ. No. 143), SEQ. No. 144, SEQ. No. 145, SEQ. No. 146, SEQ. No. 147; SEQ. No. 148, SEQ. No. 149, and SEQ. No. 150;

which encode a fusion protein having an amino acid sequence corresponding to amino acid sequences selected from the group consisting of amino acid sequences, respectively:

SEQ. No. 1, SEQ. No. 2, SEQ. No. 3, SEQ. No. 4, SEQ. No. 5, SEQ. No. 6, SEQ. No. 7, SEQ. No. 8, SEQ. No. 9, SEQ. No. 10, SEQ. No. 11, SEQ. No. 12, SEQ. No. 13, SEQ. No. 14, SEQ. No. 15, SEQ. No. 16, SEQ. No. 17, SEQ. No. 18, SEQ. No. 19, SEQ. No. 20, SEQ. No. 21, SEQ. No. 22), SEQ. No. 23, SEQ. No. 24, SEQ. No. 25, SEQ. No. 26, SEQ No. 93, SEQ. No. 94, SEQ. No. 95, SEQ. No. 96, SEQ. No. 97, SEQ. No. 98, SEQ. No. 99, SEQ. No. 100, SEQ. No. 101, SEQ. No. 102, SEQ. No. 103, SEQ. No. 104, SEQ. No. 105, SEQ. No. 106, SEQ. No. 107, SEQ. No. 108, SEQ. No. 109, SEQ. No. 110, SEQ. No. 111, SEQ. No. 112, SEQ. No. 113, SEQ. No. 114, SEQ. No. 115, SEQ. No. 116, SEQ. No. 117 and SEQ. No. 118, SEQ. No. 119, SEQ. No. 120 and SEQ. No. 121.

In a preferred embodiment, the invention provides also an expression vector suitable for transformation of *E. coli*, comprising the polynucleotide sequence selected from the group of polynucleotide sequences SEQ. No. 67 to SEQ. No. 92 and SEQ. No. 122 to SEQ. No. 150 indicated above, as well as *E. coli* cells transformed with such an expression vector.

Transformation, i.e. introduction of a DNA sequence into bacterial host cells, particularly *E. coli*, is usually performed on the competent cells, prepared to take up the DNA for example by treatment with calcium ions at low temperature (4°C), and then subjecting to the heat-shock (at 37-42°C) or by electroporation. Such techniques are well known and are usually determined by the manufacturer of the expression system.

The procedure of overexpression of fusion proteins of the invention in *E. coli* expression system will be further described below.

The invention also provides a pharmaceutical composition containing the fusion protein of the invention as defined above as an active ingredient and a suitable pharmaceutically acceptable carrier, diluent and conventional auxiliary components.

The pharmaceutical composition will contain an effective amount of the fusion protein of the invention and pharmaceutically acceptable auxiliary components dissolved or dispersed in a carrier or diluent, and preferably will

be in the form of a pharmaceutical composition formulated in a unit dosage form or formulation containing a plurality of doses.

Pharmaceutical forms and methods of their formulation as well as other components, carriers and diluents are known to the skilled person and described in the literature. For example, they are described in the monograph Remington's Pharmaceutical Sciences, ed. 20, 2000, Mack Publishing Company, Easton, USA.

The terms "pharmaceutically acceptable carrier, diluent, and auxiliary ingredient" comprise any solvents, dispersion media, surfactants, antioxidants, stabilizers, preservatives (e.g. antibacterial agents, antifungal agents), isotoning agents, known in the art. The pharmaceutical composition of the invention may contain various types of carriers, diluents and excipients, depending on the chosen route of administration and desired dosage form, such as liquid, solid and aerosol forms for oral, parenteral, inhaled, topical, and whether that selected form must be sterile for administration route such as by injection.

The preferred route of administration of the pharmaceutical composition according to the invention is parenteral, including injection routes such as intravenous, intramuscular, subcutaneous, intraperitoneal, intratumourous, or by single or continuous intravenous infusions.

In one embodiment, the pharmaceutical composition of the invention may be administered by injection directly to the tumour. In another embodiment, the pharmaceutical composition of the invention may be administered intravenously. In yet another embodiment, the pharmaceutical composition of the invention can be administered subcutaneously or intraperitoneally.

A pharmaceutical composition for parenteral administration may be a solution or dispersion in a pharmaceutically acceptable aqueous or non-aqueous medium, buffered to an appropriate pH and isoosmotic with body fluids, if necessary, and may also contain antioxidants, buffers, bacteriostatic agents and soluble substances, which make the composition compatible with the tissues or blood of recipient. Other components, which may included in the composition, are for example water, alcohols such as ethanol, polyols such as glycerol, propylene glycol, liquid polyethylene glycol, lipids such as

triglycerides, vegetable oils, liposomes. Proper fluidity and the particles size of the substance may be provided by coating substances, such as lecithin, and surfactants, such as hydroxypropylcelulose polysorbates, and the like. Suitable isotoning agents for liquid parenteral compositions are, for example, sugars such as glucose, and sodium chloride, and combinations thereof.

Alternatively, the pharmaceutical composition for administration by injection or infusion may be in a powder form, such as a lyophilized powder for reconstitution immediately prior to use in a suitable carrier such as, for example, sterile pyrogen-free water.

The pharmaceutical composition of the invention for parenteral administration may also have the form of nasal administration, including solutions, sprays or aerosols. Preferably, the form for intranasal administration will be an aqueous solution and will be isotonic or buffered o maintain the pH from about 5.5 to about 6.5, so as to maintain a character similar to nasal secretions. Moreover, it will contain preservatives or stabilizers, such as in the well-known intranasal preparations.

The composition may contain various antioxidants which delay oxidation of one or more components. Furthermore, in order to prevent the action of microorganisms, the composition may contain various antibacterial and antifungal agents, including, for example, and not limited to, parabens, chlorobutanol, thimerosal, sorbic acid, and similar known substances of this type.

In general, the pharmaceutical composition of the invention can include, for example at least about 0.01 wt% of active ingredient. More particularly, the composition may contain the active ingredient in the amount from 1% to 75% by weight of the composition unit, or for example from 25% to 60% by weight, but not limited to the indicated values.

The actual amount of the dose of the composition according to the present invention administered to patients, including man, will be determined by physical and physiological factors, such as body weight, severity of the condition, type of disease being treated, previous or concomitant therapeutic interventions, the patient and the route of administration. A suitable unit dose, the total dose and the concentration of active ingredient in the composition is to be determined by the treating physician.

The composition may for example be administered at a dose of about 1 microgram/kg of body weight to about 1000 mg/kg of body weight of the patient, for example in the range of 5 mg/kg of body weight to 100 mg/kg of body weight or in the range of 5 mg/kg of body weight to 500 mg/kg of body weight.

The fusion protein and the compositions containing it exhibit anticancer or antitumor and can be used for the treatment of cancer diseases.

The invention also provides the use of th fusion protein of the invention as defined above for treating cancer diseases in mammals, including humans.

The invention also provides a method of treating cancer diseases in mammals, including humans, comprising administering to a subject in need of such treatment an anticancer effective amount of the fusion protein of the invention as defined above, optionally in the form of appropriate pharmaceutical composition.

The fusion protein of the invention can be used for the treatment of hematologic malignancies, such as leukaemia, granulomatosis, myeloma and other hematologic malignancies. The fusion protein can also be used for the treatment of solid tumours, such as breast cancer, lung cancer, including non-small cell lung cancer, colon cancer, pancreatic cancer, ovarian cancer, bladder cancer, prostate cancer, kidney cancer, brain cancer, and the like.

Appropriate route of administration of the fusion protein in the treatment of cancer will be in particular parenteral route, which consists in administering the fusion protein of the invention in the form of injections or infusions, in the composition and form appropriate for this administration route.

The invention will be described in more detail in the following general procedures and examples of specific fusion proteins.

General procedure for overexpression of the fusion protein

Preparation of plasmid

Amino acid sequence of the target fusion protein was used as a template to generate a DNA sequence encoding it, comprising codons optimized for expression in *Escherichia coli*. Such a procedure allows to increase the efficiency of a further step of target protein synthesis in *Escherichia coli*.

Resulting nucleotide sequence was then automatically synthesized. Additionally, the cleavage sites of restriction enzymes NdeI (at the 5'-end of leading strand) and XhoI (at the 3'-end of leading strand) were added to the resulting gene encoding the target protein. These were used to clone the gene into the vector pET28a (Novagen). They may be also be used for cloning the gene encoding the protein to other vectors. Target protein expressed from this construct was equipped at the N-terminus with a polyhistidine tag (six histidines), preceded by a site recognized by thrombin, which subsequently served to its purification via affinity chromatography. The correctness of the resulting construct was confirmed firstly by restriction analysis of isolated plasmids using the enzymes NdeI and XhoI, followed by automatic sequencing of the entire reading frame of the target protein. The primers used for sequencing were complementary to the sequences of T7 promoter (5'-TAATACGACTCACTATAGG-3') and T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3 ') present in the vector.

Resulting plasmid was used for overexpression of the target fusion protein in a commercial *E. coli* strain, which was transformed according to the manufacturer's recommendations. Colonies obtained on the selection medium (LB agar, kanamycin 50 µg/ml, 1% glucose) were used for preparing an overnight culture in LB liquid medium supplemented with kanamycin (50 µg/ml) and 1% glucose. After about 15h of growth in shaking incubator, the cultures were used to inoculate the appropriate culture.

Overexpression and purification of fusion proteins - general procedure A

LB medium with kanamycin (30 µg/ml) and 100 uM zinc sulfate was inoculated with overnight culture. The culture was incubated at 37°C until the optical density (OD) at 600 nm reached 0.60-0.80. Then IPTG was added to the final concentration in the range of 0.25 -1mM. After incubation (3.5 - 20h) with shaking at 25°C the culture was centrifuged for 25 min at 6,000 g.

Bacterial pellets were resuspended in a buffer containing 50 mM KH_2PO_4, 0.5 M NaCl, 10 mM imidazole, pH 7.4. The suspension was sonicated on ice for 8 minutes (40% amplitude, 15-second pulse, 10 s interval). The resulting extract was clarified by centrifugation for 40 minutes at 20.000 g, 4°C. Ni-Sepharose (GE Healthcare) resin was pre-treated by equilibration with buffer, which was

used for preparation of the bacterial cells extract. The resin was then incubated overnight at 4°C with the supernatant obtained after centrifugation of the extract. Then it was loaded into chromatography column and washed with 15 to 50 volumes of buffer 50 mM KH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4. The obtained protein was eluted from the column using imidazole gradient in 50 mM KH_2PO_4 buffer with 0.5 M NaCl, pH 7.4. Obtained fractions were analyzed by SDS-PAGE. Appropriate fractions were combined and dialyzed overnight at 4°C against 50 mM Tris buffer, pH 7.2, 150 mM NaCl, 500 mM L-arginine, 0.1 mM $ZnSO_4$, 0.01% Tween 20, and at the same time Histag was cleaved with thrombin (1:50). After the cleavage, thrombin was separated from the target fusion protein using Benzamidine Sepharose™ resin. The purity of the product was analyzed by SDS-PAGE electrophoresis (Maniatis et al, Molecular Cloning. Cold Spring Harbor, NY, 1982).

Overexpression and purification of fusion proteins - general procedure B

LB medium with kanamycin (30 µg/ml) and 100 µM zinc sulfate was inoculated with overnight culture. Cultures were incubated at 37°C until optical density (OD) at 600 nm reached 0.60-0.80. Then IPTG was added to the final concentration in the range 0.5 -1mM. After 20h incubation with shaking at 25°C the culture was centrifuged for 25 min at 6,000 g.

Bacterial cells after overexpression were disrupted in a French Press in a buffer containing 50 mM KH_2PO_4, 0.5 M NaCl, 10 mM imidazole, 5mM beta-mercaptoethanol, 0,5mM PMSF (phenylmethylsulphonyl fluoride), pH 7.8. Resulting extract was clarified by centrifugation for 50 minutes at 8.000 g. The Ni-Sepharose resin was incubated overnight with the obtained supernatant. Then the resin with bound protein was packed into the chromatography column. To wash-out the fractions containing non-binding proteins, the column was washed with 15 to 50 volumes of buffer 50 mM KH_2PO_4, 0.5 M NaCl, 10 mM imidazole, 5mM beta-mercaptoethanol, 0,5mM PMSF (phenylmethylsulphonyl fluoride), pH 7.8. Then, to wash-out the majority of proteins binding specifically with the bed, the column was washed with a buffer containing 50 mM KH_2PO_4, 0.5 M NaCl, 500 mM imidazole, 10% glycerol, 0,5mM PMSF, pH 7.5. Obtained fractions were analyzed by SDS-PAGE (Maniatis et al, Molecular Cloning. Cold Spring Harbor, NY, 1982). The fractions containing the target protein were combined and cleaved with

thrombin (1U per 4 mg of protein, 8h at 16°C) to remove polyhistidine tag. Then the fractions were dialyzed against formulation buffer (500 mM L-arginine, 50 mM Tris, 2,5 mM $ZnSO_4$, pH 7.4).

Characterization of fusion proteins using 2-D electrophoresis

In order to further characterize obtained proteins and to select precisely chromatographic conditions, isoelectric points of the proteins were determined. For this purpose, two-dimensional electrophoresis (2-D) method was used, in two stages according to the following schedule.

Step 1. Isoelectrofocusing of proteins in a pH gradient and denaturing conditions.

Protein preparations at concentrations of 1 - 2 mg/ml were precipitated by mixing in a 1:1 ratio with a precipitation solution containing 10% trichloroacetic acid and 0.07% beta-mercaptoethanol in acetone. The mixture was incubated for 30 min at -20°C and then centrifuged for 25 min at 15,000 g and 4°C. The supernatant was removed and the pellet was washed twice with cold acetone with 0.07% beta-mercaptoethanol. Then the residues of acetone were evaporated until no detectable odour. The protein pellet was suspended in 250 ml of rehydration buffer 8M urea, 1% CHAPS, 15 mM DTT, 0.5% ampholyte (GE Healthcare) with a profile of pH 3-11 or 6-11, depending on the strip subsequently used. The protein solution was placed in a ceramic chamber for isoelectrofocusing, followed by 13 cm DryStrip (GE Healthcare) with appropriate pH profile (3-11 or 6-11). The whole was covered with a layer of mineral oil. The chambers were placed in the Ettan IPGphor III apparatus, where isoelectrofocusing was conducted according to the following program assigned to the dimensions of the strip and the pH profile:

16h dehydration at 20° C.

Focusing in the electric field at a fixed pH gradient

Time	Voltage
1h	500 V
1h	gradient 500 - 1000 V
2h 30min	gradient 1000 - 8000 V
30 min	8000 V

Then, the strip containing the focused proteins was washed for 1 min in deionised water, stained with Coomassie Brilliant and then decolorized and archived as an image to mark the location of proteins. Discoloured strip was equilibrated 2 x 15 min with a buffer of the following composition: 50mM Tris-HCl pH 8.8, 6M urea, 1% DTT, 2% SDS, 30% glycerol.

Step 2. Separation in a second direction by SDS-PAGE.

The strip was placed over the 12.5% polyacrylamide gel containing a single well per standard size and then separation was performed in an apparatus for SDS-PAGE, at a voltage of 200V for 3 hours. The gel was stained with Coomassie Brilliant then archived with the applied scale. Proteins were identified by determining its weight on the basis of the standard of size, and its IPI was read for the scale of 6-11 on the basis of the curves provided by the manufacturer (GE Healthcare) (ratio of pH to % of length of the strip from the end marked as anode) or a scale of 3-11 on the basis of the curve determined experimentally by means of isoelectrofocusing calibration kit (GE Healthcare).

Representative examples of the fusion proteins of the invention are described below.

Example 1. The fusion protein of SEQ. No. 1

The protein of SEQ. No. 1 is a fusion protein having the length of 194 amino acids and the mass of 22.7 kDa, in which at the N-terminus of TRAIL121-281 sequence the 16-amino acid peptide derived from the BH3 domain of Bax protein (SEQ. No. 30) is attached as the effector peptide. At the C-terminus of the 16-amino acid sequence of the effector peptide there is attached the polyarginine sequence of 7 Arg/R residues. Polyarginine sequence aids in penetration of the cell membrane and transportation of the fusion protein into the cell. Between the polyarginine sequence and TRAIL domain there are incorporated sequentially next to each other sequences recognized by urokinase uPA (SEQ. No. 52) and metalloprotease MMP (SEQ. No. 51), due to which the effector peptide upon internalization of the fusion protein will be cleaved in the tumour environment.

Structure of the fusion protein is presented schematically in Fig. 1 and its amino acid sequence and the DNA encoding sequence comprising codons

optimized for expression in *E. coli* are, respectively, SEQ. No. 1 and SEQ. No. 67 as shown below.

Amino acid sequence: SEQ. No. 1

1 KKLSECLKRI GDELDSRRRR RRRRVVRPLG LAGRVAAHIT GTRGRSNTLS
51 SPNSKNEKAL GRKINSWESS RSGHSFLSNL HLRNGELVIH EKGFYYIYSQ
101 TYFRFQEEIK ENTKNDKQMV QYIYKYTSYP DPILLMKSAR NSCWSKDAEY
151 GLYSIYQGGI FELKENDRIF VSVTNEHLID MDHEASFFGA FLVG

DNA sequence: SEQ. No. 67

1 GCCCACCAGA AATGCACCAA AAAAGCTGGC TTCATGATCC ATATCAATCA
GATGTTCATT GGTCACGCTC ACAAAAATGC GATCATTTTC TTTCAGTTCA
101 AAAATGCCAC CCTGATAAAT GCTATACAGG CCATATTCTG CATCTTTGCT
CCAACAGCTA TTACGTGCGC TTTTCATCAG CAGAATCGGA TCCGGATAGC
201 TGGTATATTT ATAAATGTAC TGCACCATTT GTTTATCATT TTTGGTATTT
TCTTTAATTT CTTCCTGAAA GCGAAAATAG GTCTGGCTAT AAATATAATA
301 AAAGCCTTTT TCATGAATCA CCAGTTCACC ATTACGCAGA TGCAGATTGC
TCAGAAAGCT ATGACCGCTA CGGCTGCTTT CCCAGCTATT AATTTTGCGA
401 CCCAGGGCTT TTTCATTTTT GCTATTCGGG CTGCTCAGGG TATTGCTACG
ACCACGGGTG CCGGTAATAT GTGCTGCAAC ACGACCTGCC AGACCCAGCG
501 GACGAACAAC ACGACGACGG CGACGACGAC GACGGCTATC CAGTTCATCA
CCAATACGTT TCAGGCATTC GCTCAGTTTT TT

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strains BL21 (DE3) and Tuner (DE3) pLysS, both from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 2. The fusion protein of SEQ. No. 2

The fusion protein of SEQ. No. 2 is a protein having the length of 193 amino acids and the mass of 22.5 kDa, in which at the N-terminus of 121-281TRAIL sequence the 16-amino acid peptide derived from the Bid protein (SEQ. No.

31) is attached as the effector peptide. Additionally, to the C-terminus of the effector protein there is attached polyarginine sequence consisting of seven Arg residues. Polyarginine sequence aids in penetration of the cell membrane and transportation of the fusion protein into the cell. Between the polyarginine sequence and the sequence of TRAIL sequences recognized by metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52) are incorporated sequentially next to each other, due to which upon internalization of the fusion protein the effector peptide will be cleaved in the tumour environment.

Structure of the fusion protein is shown schematically in Fig. 1 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 2 and SEQ. No. 68 as shown below.

Amino acid sequence: SEQ. No. 2

1 RNIARHLAQV GDSMDRRRRR RRRVVRPLGL AGRVAAHITG TRGRSNTLSS
51 PNSKNEKALG RKINSWESSR SGHSFLSNLH LRNGELVIHE KGFYYIYSQT
101 YFRFQEEIKE NTKNDKQMVQ YIYKYTSYPD PILLMKSARN SCWSKDAEYG
151 LYSIYQGGIF ELKENDRIFV SVTNEHLIDM DHEASFFGAF LVG

DNA sequence: SEQ. No. 68

1 CGTAATATTG CACGTCATCT GGCACAGGTT GGTGATAGCA TGGACCGTCG
TCGTCGTCGC CGTCGTCGTG TTGTTCGTCC GCTGGGTCTG GCAGGTCGTG
101 TTGCAGCACA TATTACCGGC ACCCGTGGTC GTAGCAATAC CCTGAGCAGC
CCGAATAGCA AAAATGAAAA AGCCCTGGGT CGCAAAATTA ATAGCTGGGA
201 AAGCAGCCGT AGCGGTCATA GCTTTCTGAG CAATCTGCAT CTGCGTAATG
GTGAACTGGT GATTCATGAA AAAGGCTTTT ATTATATTTA TAGCCAGACC
301 TATTTTCGCT TTCAGGAAGA AATTAAAGAA AATACCAAAA ATGATAAACA
AATGGTGCAG TACATTTATA AATATACCAG CTATCCGGAT CCGATTCTGC
401 TGATGAAAAG CGCACGTAAT AGCTGTTGGA GCAAAGATGC AGAATATGGC
CTGTATAGCA TTTATCAGGG TGGCATTTTT GAACTGAAAG AAAATGATCG
501 CATTTTTGTG AGCGTGACCA ATGAACATCT GATTGATATG GATCATGAAG
CCAGCTTTTT TGGTGCATTT CTGGTGGGC

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding

sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strain BL21 (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 3. The fusion protein of SEQ. No. 3

The fusion protein of SEQ. No. 3 is a protein having the length of 303 amino acids and the mass of 34.2 kDa, in which at the C-terminus of the 121-281TRAIL sequence the homologue of ribonuclease RNase A (SEQ. No. 32) is attached as an effector peptide. Between the polyarginine sequence and the sequence of TRAIL sequences recognized by metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52) are sequentially incorporated next to each other, due to which upon internalization of the fusion protein the effector peptide will be cleaved in the tumour environment.

The protein also contains, between the TRAIL domain sequence and the sequence of cleavage sites, flexible glycine-serine linker GGSG (SEQ. No. 57). Furthermore, at the C-terminus of the effector peptide, the protein contains the sequence KEDL (SEQ. No. 56) directing to the endoplasmic reticulum, being also a C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Fig. 1 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 3 and SEQ. No. 69 as shown below.

Amino acid sequence: SEQ. No. 3

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGSGPLGLA GRVVRKETAA AKFERQHMDS STSAASSSNY
201 CNQMMKSRNL TKDRCKPVNT FVHESLADVQ AVCSQKNVAC KNGQTNCYQS
251 YSTMSITDCR ETGSSKYPNC AYKTTQANKH IIVACEGNPY VPVHFDASVK
301 EDL

DNA sequence: SEQ. No. 69

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCCCT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAGG AAGAAATTAA AGAAAATACC AAAAATGACA
AACAAATGGT GCAGTATATC TACAAATACA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGCCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTA GCGGTCCGCT
501 GGGTCTGGCA GGTCGTGTTG TTCGTAAAGA AACCGCAGCA GCCAAATTTG
AACGTCAGCA CATGGATAGC AGCACCAGCG CAGCAAGCAG CAGCAATTAT
601 TGCAATCAGA TGATGAAAAG CCGCAATCTG ACCAAAGATC GTTGTAAACC
GGTGAATACC TTTGTTCATG AAAGCCTGGC AGATGTTCAG GCAGTTTGCA
701 GCCAGAAAAA TGTGGCCTGT AAAAATGGTC AGACCAATTG CTATCAGAGC
TATAGCACCA TGAGCATTAC CGATTGTCGT GAAACCGGTA GCAGCAAATA
801 TCCGAATTGC GCCTATAAAA CCACCCAGGC CAATAAACAT ATTATTGTGG
CCTGTGAAGG CAATCCGTAT GTTCCGGTTC ATTTTGATGC CAGCGTGAAA
901 GAAGATCTG

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Over-expression was performed according to the general procedure B, using *E. coli* strain BL21 (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 4. The fusion protein of SEQ. No. 4

The protein of SEQ. No. 4 is a fusion protein having the length of 293 amino acids and the mass of 33.2 kDa, in which at the C-terminus of TRAIL 121-281 sequence the homologue of ribonuclease RNase A (SEQ. No. 32) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL there is a flexible glycine-serine linker GGGSGGGS (SEQ. No. 63).

Structure of the fusion protein is shown schematically in Fig. 1 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 4 and SEQ. No. 70 as shown below.

Amino acid sequence: SEQ. No. 4

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGGSGGGSK ETAAAKFERQ HMDSSTSAAS SSNYCNQMMK
201 SRNLTKDRCK PVNTFVHESL ADVQAVCSQK NVACKNGQTN CYQSYSTMSI
251 TDCRETGSSK YPNCAYKTTQ ANKHIIVACE GNPYVPVHFD ASV

DNA sequence: SEQ. No. 70

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAATACC AAAAATGATA
AGCAGATGGT GCAGTATATC TATAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCGGTGG
501 TGGTAGTAAA GAAACCGCAG CAGCAAAATT TGAACGTCAG CACATGGATA
GCAGCACCAG CGCAGCAAGC AGCAGCAATT ATTGTAATCA GATGATGAAA
601 AGCCGCAATC TGACCAAAGA TCGTTGTAAA CCGGTGAATA CCTTTGTTCA
TGAAAGCCTG GCAGATGTTC AGGCAGTTTG TAGCCAGAAA AATGTTGCCT
701 GTAAAAATGG TCAGACCAAT TGCTATCAGA GCTATAGCAC CATGAGCATT
ACCGATTGTC GTGAAACCGG TAGCAGCAAA TATCCGAATT GTGCATATAA
801 AACCACCCAG GCCAATAAAC ATATTATTGT TGCCTGTGAA GGCAATCCGT
ATGTTCCGGT TCATTTTGAT GCAAGCGTT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was

carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E. coli* strains BL21DE3pLysSRIL from Stratagene and Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 5. The fusion protein of SEQ. No. 5

The protein of SEQ. No. 5 is a fusion protein having the length of 283 amino acids and the mass of 31 kDa, in which at the C-terminus of 121-281TRAIL sequence the sequence of cytochrome C (SEQ. No. 33) is attached as an effector peptide. Between the sequence of TRAIL domain and the sequence of effector protein the sequences recognized by metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52) are incorporated sequentially next to each other, due to which upon internalization of the fusion protein the effector peptide will be cleaved in the tumour environment. The protein also contains, between the TRAIL domain sequence and the sequence of cleavage sites, the flexible glycine-serine linker GGSG (SEQ. No. 57). Furthermore, at the C-terminus of the effector peptide the protein contains the sequence KEDL (SEQ. No. 56) directing to the endoplasmic reticulum, which is a C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Fig. 1 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 5 and SEQ. No. 71 as shown below.

Amino acid sequence: SEQ. No. 5

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGSGPLGLA GRVVRGDVEK GKKIFIMKCS QCHTVEKGGK
201 HKTGPNLHGL FGRKTGQAPG YSYTAANKNK GIIWGEDTLM EYLENPKKYI
251 PGTKMIFVGI KKKEERADLI AYLKKATNEK EDL

DNA sequence: SEQ. No. 71

1 CAGATCTTCT TTTTCATTGG TGGCTTTTTT CAGATAGGCA ATCAGATCTG
CGCGTTCTTC TTTTTTTTTA ATGCCCACAA AAATCATTTT CGTACCCGGA
101 ATATATTTTT TCGGATTTTC CAGATATTCC ATCAGGGTAT CTTCACCCCA
AATAATGCCT TTGTTTTTAT TGGCTGCGGT ATAGCTATAA CCCGGTGCCT
201 GACCGGTTTT ACGACCAAAC AGACCATGCA GATTCGGACC GGTTTTATGT
TTGCCACCTT TTTCAACGGT ATGACACTGG CTGCATTTCA TAATAAAAAT
301 TTTTTTGCCT TTTTCCACAT CACCACGAAC AACACGACCT GCCAGACCCA
GCGGACCGCT ACCACCACCA ACCAGAAATG CACCAAAAAA GCTGGCTTCA
401 TGATCCATAT CAATCAGATG TTCATTGGTC ACGCTCACAA AAATGCGATC
ATTTTCTTTC AGTTCAAAAA TGCCACCCTG ATAAATGCTA TACAGGCCAT
501 ATTCTGCATC TTTGCTCCAA CAGCTATTAC GTGCGCTTTT CATCAGCAGA
ATCGGATCCG GATAGCTGGT ATATTTATAA ATGTACTGCA CCATTTGTTT
601 ATCGTTTTTG GTATTTTCTT TAATTTCTTC CTGAAAGCGA AAATAGGTCT
GGCTATAAAT ATAATAAAAG CCTTTTTCAT GAATCACCAG TTCACCATTA
701 CGCAGATGCA GATTGCTCAG AAAGCTATGA CCGCTACGGC TGCTTTCCCA
GCTATTAATT TTGCGACCCA GGGCTTTTTC ATTTTTGCTA TTCGGGCTGC
801 TCAGGGTATT GCTACGACCA CGGGTGCCGG TAATATGTGC TGCAACACGC AT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 6. The fusion protein of SEQ. No. 6

The protein of SEQ. No. 6 is a fusion protein having the length of 407 amino acids and the mass of 45.2 kDa, in which at the C-terminus of 121-281TRAIL sequence the sequence of cytochrome C (SEQ. No. 33) is attached as an effector peptide. Between the sequence of TRAIL domain and the effector peptide there are the sequence recognized by furin (SEQ. No. 53) and the translocation domain from *Pseudomonas aeruginosa* (SEQ. No. 54). The protein also contains flexible linkers: between the sequence of TRAIL sequence and the sequence of cleavage site recognized by furin there is the flexible glycine-serine linker GGGS (SEQ. No. 58), between the sequence of

cleavage site recognized by furin and the translocation domain from *Pseudomonas aeruginosa* the flexible glycine-serine linker ASGG (SEQ. No. 65), and between the sequence of translocation domain and the sequence of cytochrome C the flexible glycine-serine linker GGGSGGG (SEQ. No. 62). Furthermore, at the C-terminus of the effector peptide domain, the protein contains a sequence KEDL (SEQ. No. 56) directing to the endoplasmic reticulum, which is a C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Fig. 2 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 6 and SEQ. No. 72 as shown below.

Amino acid sequence: SEQ. No. 6

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGGSRKKRA SGGPEGGSLA ALTAHQACHL PLETFTRHRQ
201 PRGWEQLEQC GYPVQRLVAL YLAARLSWNQ VDQVIANALA SPGSGGDLGE
251 AIRESPEQAR LALTLAAAES ERFVRQGTGN DEAGAANGPA DGGGSGGGMG
301 DVEKGKKIFI MKCSQCHTVE KGGKHKTGPN LHGLFGRKTG QAPGYSYTAA
351 NKNKGIIWGE DTLMEYLENP KKYIPGTKMI FVGIKKKEER ADLIAYLKKA
401 TNEKDEL

DNA sequence: SEQ. No. 72

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAATACC AAAAATGACA
AACAAATGGT GCAGTATATC TATAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCCGTAA

501 AAAACGTGCA AGCGGTGGTC CGGAAGGTGG TAGCCTGGCA GCACTGACCG
CACATCAGGC ATGTCATCTG CCGCTGGAAA CCTTTACCCG TCATCGTCAG
601 CCTCGTGGTT GGGAACAGCT GGAACAGTGT GGTTATCCGG TTCAGCGTCT
GGTTGCACTG TATCTGGCAG CACGTCTGAG CTGGAATCAG GTTGATCAGG
701 TTATTGCAAA TGCACTGGCA AGTCCGGGTA GCGGTGGTGA TCTGGGTGAA
GCAATTCGTG AAAGTCCGGA ACAGGCACGT CTGGCACTGA CCCTGGCAGC

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 7. The fusion protein of SEQ. No. 7

The protein of SEQ. No. 7 is a fusion having the length of 409 amino acids and the mass of 46.1 kDa, in which at the N-terminus of the sequence of TRAIL114-281 the sequence of granzyme B (SEQ. No. 34) is attached as an effector peptide. Between the sequence of TRAIL domain and the sequence of the effector peptide granzyme B there is the sequence of the furin cleavage site (SEQ. No. 53), additionally flanked by the flexible glycine-serine linkers GGGGS (SEQ. No. 59).

Structure of the fusion protein is shown schematically in Fig. 2 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 7 and SEQ. No. 73 as shown below.

Amino acid sequence: SEQ. No. 7

1 IIGGHVAKPH SRPYMAYLMI WDQKSLKRCG GFLIRDDFVL TAAHCWGSSI
51 NVTLGAHNIK EQEPTQQFIP VKRAIPHPAY NPKNFSNDIM LLQLERKAKR
101 TRAVQPLRLP SNKAQVKPGQ TCSVAGWGQT APLGKHSHTL QEVKMTVQED
151 RKCESDLRHY YDSTIELCVG DPEIKKTSFK GDSGGPLVCN KVAQGIVSYG
201 RNNGMPPRAC TKVSSFVHWI KKTMKRYGGG GSRKKRGGGG SVRERGPQRV
251 AAHITGTRGR SNTLSSPNSK NEKALGRKIN SWESSRSGHS FLSNLHLRNG
301 ELVIHEKGFY YIYSQTYFRF QEEIKENTKN DKQMVQYIYK YTSYPDPILL

351 MKSARNSCWS KDAEYGLYSI YQGGIFELKE NDRIFVSVTN EHLIDMDHEA
401 SFFGAFLVG

DNA sequence: SEQ. No. 73

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCCCT GGGTCGTAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGCGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAGG AAGAAATTAA AGAAAATACC AAAAATGATA
AACAAATGGT GCAGTATATC TATAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCCGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCCGTAA
501 AAAACGTGGT GGTGGCGGTT CTATTATTGG TGGTCATGTT GCAAAACCGC
ATAGCCGTCC GTATATGGCA TATCTGATGA TTTGGGATCA GAAAAGCCTG
601 AAACGTTGTG GTGGCTTTCT GATTCGTGAT GATTTTGTTC TGACCGCAGC
ACATTGTTGG GGTAGCAGCA TTAATGTTAC CCTGGGTGCC CATAATATTA
701 AAGAACAGGA ACCGACCCAG CAGTTTATTC CGGTTAAACG TGCAATTCCG
CATCCGGCAT ATAATCCGAA AAATTTTAGC AATGATATCA TGCTGCTGCA
801 GCTGGAACGT AAAGCAAAAC GTACCCGTGC AGTTCAGCCG CTGCGTCTGC
CGAGCAATAA AGCACAGGTT AAACCGGGTC AGACCTGTAG CGTTGCAGGT
901 TGGGGTCAGA CCGCACCGCT GGGTAAACAT TCTCATACCC TGCAAGAGGT
TAAAATGACC GTCCAAGAGG ATCGTAAATG CGAAAGCGAT CTGCGCCATT
1001 ATTATGATAG CACCATTGAA CTGTGTGTGG GCGATCCGGA AATCAAAAAA
ACCAGCTTTA AAGGTGATAG CGGTGGTCCG CTGGTTTGTA ATAAAGTTGC
1101 CCAGGGTATT GTTAGCTATG GTCGTAATAA TGGTATGCCG CCGCGTGCAT
GTACCAAAGT TAGCAGCTTT GTGCATTGGA TTAAAAAAAC GATGAAACGC
1201 TATAAAGATG AACTG

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using

E. coli strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 8. The fusion protein of SEQ. No. 8

The protein of SEQ. No. 8 is a fusion protein having the length of 405 amino acids and the mass of 45.7 kDa, in which at the C-terminus of TRAIL 121-281 sequence the sequence of granzyme B (SEQ. No. 34) is attached as an effector peptide. Between the sequence of TRAIL and the sequence of the effector peptide there is the sequence of furin cleavage site (SEQ. No. 53), additionally separated from the sequences of both granzyme B and TRAIL with flexible glycine-serine linkers GGGS (Sekw. Nr 58) and GGGGS (SEQ. No. 59), respectively. Furthermore, at the C-terminus of the effector peptide, the protein contains the sequence KDEL directing the endoplasmic reticulum, which is the C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Fig. 2 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 8 and SEQ. No. 74 as shown below.

Amino acid sequence: SEQ. No. 8

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGGSRKKRG GGGSIIGGHV AKPHSRPYMA YLMIWDQKSL
201 KRCGGFLIRD DFVLTAAHCW GSSINVTLGA HNIKEQEPTQ QFIPVKRAIP
251 HPAYNPKNFS NDIMLLQLER KAKRTRAVQP LRLPSNKAQV KPGQTCSVAG
301 WGQTAPLGKH SHTLQEVKMT VQEDRKCESD LRHYYDSTIE LCVGDPEIKK
351 TSFKGDSGGP LVCNKVAQGI VSYGRNNGMP PRACTKVSSF VHWIKKTMKR
401 YKDEL

DNA sequence: SEQ. No. 74

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA

201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAACACC AAAAATGATA
AACAAATGGT GCAGTATATT TACAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCCGTAA
501 AAAACGTGGT GGTGGCGGTA GTATTATTGG TGGTCATGTT GCAAAACCGC
ATAGCCGTCC GTATATGGCA TATCTGATGA TTTGGGATCA GAAAAGCCTG
601 AAACGTTGTG GTGGTTTTCT GATTCGTGAT GATTTTGTTC TGACCGCAGC
ACATTGTTGG GGTAGCAGCA TTAATGTTAC CCTGGGTGCC CATAATATTA
701 AAGAACAAGA ACCGACCCAG CAGTTTATTC CGGTTAAACG TGCAATTCCG
CATCCGGCAT ATAATCCGAA AAATTTTAGC AATGATATTA TGCTGCTGCA
801 GCTGGAACGC AAAGCAAAAC GTACCCGTGC AGTTCAGCCG CTGCGTCTGC
CGAGCAATAA AGCACAGGTT AAACCGGGTC AGACCTGTAG CGTTGCAGGT
901 TGGGGTCAGA CCGCACCGCT GGGTAAACAT TCACATACCC TGCAAGAGGT
GAAAATGACC GTTCAAGAGG ATCGTAAATG CGAAAGCGAT CTGCGCCATT
1001 ATTATGATAG CACCATTGAA CTGTGTGTTG GTGATCCGGA AATTAAAAAA
ACCAGCTTTA AAGGCGATAG CGGTGGTCCG CTGGTTTGTA ATAAAGTTGC
1101 ACAGGGTATT GTGAGCTATG GTCGTAATAA TGGTATGCCT CCGCGTGCAT
GTACCAAAGT TAGCAGCTTT GTGCATTGGA TTAAAAAAAC GATGAAACGC
1201 TATAAAGATG AACTG

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strain Tuner (DE3) pLysS from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 9. The fusion protein of SEQ. No. 9

The protein of SEQ. No. 9 is a fusion protein having the length of 187 amino acids and the mass of 21.9 kDa, in which at the N-terminus of TRAIL121-281 sequence the 9-amino acid peptide derived from Nur77 protein (SEQ. No. 35) is attached as an effector peptide, the polyarginine sequence consisting of seven Arg residues being additionally attached at the C-terminus of the

effector peptide. Between the effector peptide and the sequence of TRAIL there is a sequence of cleavage sites for metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Fig. 2 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 9 and SEQ. No. 75 as shown below.

Amino acid sequence: SEQ. No. 9

1 FSRSLHSLLR RRRRRRRVVR PLGLAGRVAA HITGTRGRSN TLSSPNSKNE
51 KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE
101 EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD AEYGLYSIYQ
151 GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG

DNA sequence: SEQ. No. 75

1 TTTAGCCGTA GCCTGCATAG CCTGCTGCGT CGTCGTCGTC GCCGTCGTCG
TGTTGTTCGT CCGCTGGGTC TGGCAGGTCG TGTTGCAGCA CATATTACCG
101 GCACCCGTGG TCGTAGCAAT ACCCTGAGCA GCCCGAATAG CAAAAATGAA
AAAGCCCTGG GTCGCAAAAT TAATAGCTGG GAAAGCAGCC GTAGCGGTCA
201 TAGCTTTCTG AGCAATCTGC ATCTGCGTAA TGGTGAACTG GTGATTCATG
AAAAAGGCTT TTATTATATT TATAGCCAGA CCTATTTTCG CTTTCAGGAA
301 GAAATTAAAG AAAATACCAA AAATGATAAA CAAATGGTGC AGTACATTTA
TAAATATACC AGCTATCCGG ATCCGATTCT GCTGATGAAA AGCGCACGTA
401 ATAGCTGTTG GAGCAAAGAT GCAGAATATG GCCTGTATAG CATTTATCAG
GGTGGCATTT TTGAACTGAA AGAAAATGAT CGCATTTTTG TGAGCGTGAC
501 CAATGAACAT CTGATTGATA TGGATCATGA AGCCAGCTTT TTTGGTGCAT
TTCTGGTGGG C

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strain Rosetta (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 10. The fusion protein of SEQ. No. 10

The protein of SEQ. No. 10 is a fusion protein having the length of 193 amino acids and the mass of 22.4 kDa, in which at the N-terminus of the sequence TRAIL121-281 the 16-amino acid peptide containing the BH3 domain of Bak protein (SEQ. No. 36) is attached as an effector peptide, the membrane penetrating polyarginine sequence, consisting of seven Arg residues being additionally attached at the C-terminus of the effector peptide. Between the sequence of the effector peptide and the sequence of TRAIL there are sequences of the cleavage sites for metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Fig. 2 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 10 and SEQ. No. 76 as shown below.

Amino acid sequence: SEQ. No. 10

1 GQVGRQLAII GDDINRRRRR RRRVVRPLGL AGRVAAHITG TRGRSNTLSS
51 PNSKNEKALG RKINSWESSR SGHSFLSNLH LRNGELVIHE KGFYYIYSQT
101 YFRFQEEIKE NTKNDKQMVQ YIYKYTSYPD PILLMKSARN SCWSKDAEYG
151 LYSIYQGGIF ELKENDRIFV SVTNEHLIDM DHEASFFGAF LVG

DNA sequence: SEQ. No. 76

1 GGTCAGGTTG GTCGTCAGCT GGCAATTATT GGTGATGATA TTAACCGTCG
TCGTCGTCGC CGTCGTCGTG TTGTTCGTCC GCTGGGTCTG GCAGGTCGTG
101 TTGCAGCACA TATTACCGGC ACCCGTGGTC GTAGCAATAC CCTGAGCAGC
CCGAATAGCA AAAATGAAAA AGCCCTGGGT CGCAAAATTA ATAGCTGGGA
201 AAGCAGCCGT AGCGGTCATA GCTTTCTGAG CAATCTGCAT CTGCGTAATG
GTGAACTGGT GATTCATGAA AAAGGCTTTT ATTATATTTA TAGCCAGACC
301 TATTTTCGCT TTCAGGAAGA AATTAAAGAA AATACCAAAA ATGATAAACA
AATGGTGCAG TACATTTATA AATATACCAG CTATCCGGAT CCGATTCTGC
401 TGATGAAAAG CGCACGTAAT AGCTGTTGGA GCAAAGATGC AGAATATGGC
CTGTATAGCA TTTATCAGGG TGGCATTTTT GAACTGAAAG AAAATGATCG
501 CATTTTTGTG AGCGTGACCA ATGAACATCT GATTGATATG GATCATGAAG
CCAGCTTTTT TGGTGCATTT CTGGTGGGC

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strains BL21 (DE3) and Tuner (DE3) pLysS from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above

Example 11. The fusion protein of SEQ. No. 11

The protein of SEQ. No. 11 is a fusion protein having the length of 204 amino acids and the mass of 24.3 kDa, in which at the N-terminus of the sequence of TRAIL 121-281 the BH3 domain of the PUMA/BBC3 molecule (SEQ. No. 37) is attached as an effector peptide, the polyarginine sequence comprising 9 Arg residues being additionally attached at the C-terminus of the effector peptide. Between sequence of the effector peptide and the sequence of TRAIL the construct contains also sequences of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 3 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 11 and SEQ. No. 77 as shown below.

Amino acid sequence: SEQ. No. 11

1 EEQWAREIGA QLRRMADDLN AQYERRRRRR RRRRVVRPLG LAGRVAAHIT
51 GTRGRSNTLS SPNSKNEKAL GRKINSWESS RSGHSFLSNL HLRNGELVIH
101 EKGFYYIYSQ TYFRFQEEIK ENTKNDKQMV QYIYKYTSYP DPILLMKSAR
151 NSCWSKDAEY GLYSIYQGGI FELKENDRIF VSVTNEHLID MDHEASFFGA
201 FLVG

DNA sequence: SEQ. No. 77

1 GAAGAACAGT GGGCACGTGA AATTGGTGCA CAGCTGCGTC GTATGGCAGA
TGATCTGAAT GCACAGTATG AACGTCGTCG TCGTCGCCGT CGGCGTCGTC
101 GTGTTGTTCG TCCGCTGGGT CTGGCAGGTC GTGTTGCAGC ACATATTACC
GGCACCCGTG GTCGTAGCAA TACCCTGAGC AGCCCGAATA GCAAAAATGA

201 AAAAGCACTG GGTCGCAAAA TCAATAGCTG GGAAAGCAGC CGTAGCGGTC
ATAGCTTTCT GAGCAATCTG CATCTGCGTA ATGGTGAACT GGTGATTCAT
301 GAAAAAGGCT TTTATTATAT TTATAGCCAG ACCTATTTTC GCTTTCAAGA
AGAGATTAAA GAAAATACCA AAAATGATAA ACAAATGGTG CAGTATATTT
401 ACAAATACAC CAGCTATCCG GACCCGATTC TGCTGATGAA AAGCGCACGT
AATAGCTGTT GGAGCAAAGA TGCAGAATAT GGTCTGTATA GCATTTATCA
501 GGGTGGCATC TTTGAGCTGA AAGAAAATGA TCGCATCTTT GTTAGCGTGA
CCAACGAACA TCTGATCGAT ATGGATCATG AAGCCAGCTT TTTTGGTGCA

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* strains BL21 (DE3) and Tuner (DE3) pLysS from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above

Example 12. The fusion protein of SEQ. No. 12

The protein of SEQ. No. 12 is a fusion protein having the length of 372 amino acids and the mass of 41 kDa, in which at the C-terminus of the sequence of TRAIL121-281 the PUMA protein (SEQ. No. 38) is attached as an effector peptide. Between the sequence of TRAIL the and the sequence of the effector peptide there is a sequence of cleavage sites recognized by metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52), which additionally is separated from the sequence of TRAIL by the flexible glycine-serine linker GGSGG (SEQ. No. 60). Furthermore, at the C-terminus the effector peptide comprises the KEDL sequence (SEQ. No. 56) directing to the endoplasmic reticulum, and forming a C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Fig. 3 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 12 and SEQ. No. 78 as shown below.

Amino acid sequence: SEQ. No. 12

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR

51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGSGGPLGL AGRVVRARAR QEGSSPEPVE GLARDGPRPF
201 PLGRLVPSAV SCGLCEPGLA AAPAAPTLLP AAYLCAPTAP PAVTAALGGS
251 RWPGGPRSRP RGPRPDGPQP SLSLAEQHLE SPVPSAPGAL AGGPTQAAPG
301 VRGEEEQWAR EIGAQLRRMA DDLNAQYERR RQEEQQRHRP SPWRVLYNLI
351 MGLLPLPRGH RAPEMEPNKE DL

DNA sequence: SEQ. No. 78

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATCAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAGATTAA AGAAAATACC AAAAATGATA
AACAAATGGT GCAGTACATT TACAAATATA CCAGCTATCC GGACCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT CTTTGAGCTG AAAGAAAATG
401 ATCGCATCTT TGTTAGCGTG ACCAACGAAC ATCTGATCGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTA GCGGTGGTCC
501 GCTGGGTCTG GCAGGTCGTG TTGTTCGTGC CCGTGCGCGT CAAGAAGGTA
GCAGTCCGGA ACCGGTTGAA GGTCTGGCAC GTGATGGTCC GCGTCCGTTT
601 CCGCTGGGTC GTCTGGTTCC GAGCGCAGTT AGCTGTGGTC TGTGTGAACC
GGGTCTGGCA GCCGCACCGG CAGCACCGAC ACTGCTGCCT GCAGCATATC
701 TGTGTGCACC GACCGCACCG CCTGCAGTTA CCGCAGCACT GGGTGGTAGC
CGTTGGCCTG GTGGTCCGCG TAGTCGTCCG CGTGGTCCTC GTCCGGATGG
801 TCCGCAGCCG AGCCTGAGCC TGGCAGAACA GCATCTGGAA AGTCCGGTGC
CGAGCGCACC GGGTGCACTG GCAGGCGGTC CTACACAGGC AGCACCGGGT
901 GTTCGTGGTG AAGAGGAACA GTGGGCACGT GAAATTGGTG CACAGCTGCG
TCGTATGGCA GATGATCTGA ATGCACAGTA TGAACGTCGT CGTCAAGAAG
1001 AACAGCAGCG TCATCGTCCG AGCCCGTGGC GTGTTCTGTA TAATCTGATT
ATGGGTCTGC TGCCGCTGCC TCGTGGTCAT CGTGCACCGG AAATGGAACC
1101 GAATAAAGAA GATCTG

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was

carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E. coli* strains *B.21 (DE3)* from Novagen i *BL21DE3pLysSRIL* from Stratagene. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 13. The fusion protein of SEQ. No. 13

The protein of SEQ. No. 13 is a fusion protein having the length of 493 amino acids and the mass of 53.4 kDa, in which at the C-terminus of the 121-281 TRAIL the sequence of PUMA protein (SEQ. No. 38) is attached as an effector peptide. Furthermore, between the sequence of TRAIL and the sequence of PUMA protein there is a sequence of translocation domain from *Pseudomonas aeruginosa* (SEQ. No. 54), which is further separated from the sequence of TRAIL by consecutive sequences of: flexible glycine-serine linker GGGGS (SEQ. No. 59), furin cleavage site (SEQ. No. 53) and flexible alanine-glycine-serine linker ASGG (SEQ. No. 65), and from the PUMA protein by flexible glycine-serine linker GGSGG (SEQ. No. 60). Furthermore, at the C-terminus of the effector peptide the fusion protein contains the sequence KEDL (SEQ. No. 56) directing to the endoplasmic reticulum, which is the C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Fig. 3 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 13 and SEQ. No. 79 as shown below.

Amino acid sequence: SEQ. No. 13

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGGSRKKRA SGGPEGGSLA ALTAHQACHL PLETFTRHRQ
201 PRGWEQLEQC GYPVQRLVAL YLAARLSWNQ VDQVIANALA SPGSGGDLGE
251 AIRESPEQAR LALTLAAAES ERFVRQGTGN DEAGAANGPA DGGSGGGARA
301 RQEGSSPEPV EGLARDGPRP FPLGRLVPSA VSCGLCEPGL AAAPAAPTLL
351 PAAYLCAPTA PPAVTAALGG SRWPGGPRSR PRGPRPDGPQ PSLSLAEQHL
401 ESPVPSAPGA LAGGPTQAAP GVRGEEEQWA REIGAQLRRM ADDLNAQYER

451 RRQEEQQRHR PSPWRVLYNL IMGLLPLPRG HRAPEMEPNK DEL

DNA sequence: SEQ. No. 79

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAATACC AAAAATGATA
AGCAGATGGT GCAGTATATC TATAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCCGTAA
501 AAAACGTGCA AGCGGTGGTC CGGAAGGTGG TAGCCTGGCA GCACTGACCG
CACATCAGGC ATGTCATCTG CCGCTGGAAA CCTTTACCCG TCATCGTCAG
601 CCTCGTGGTT GGGAACAGCT GGAACAGTGT GGTTATCCGG TTCAGCGTCT
GGTTGCACTG TATCTGGCAG CACGTCTGAG CTGGAATCAG GTTGATCAGG
701 TTATTGCAAA TGCACTGGCA AGTCCGGGTA GCGGTGGTGA TCTGGGTGAA
GCAATTCGTG AAAGTCCGGA ACAGGCACGT CTGGCACTGA CCCTGGCAGC
801 AGCAGAAAGC GAACGTTTTG TTCGTCAGGG CACCGGTAAT GATGAAGCCG
GTGCAGCAAA TGGTCCGGCA GATGGTGGTA GTGGTGGTGG TGCACGTGCT
901 CGTCAAGAAG GTAGCAGTCC GGAACCGGTT GAAGGTCTGG CACGTGACGG
TCCGCGTCCG TTTCCGCTGG GTCGTCTGGT TCCGAGCGCA GTTAGCTGTG
1001 GTCTGTGTGA ACCGGGTCTG GCAGCCGCAC CGGCAGCACC GACACTGCTG
CCTGCAGCAT ATCTGTGTGC ACCGACCGCA CCGCCTGCAG TTACCGCAGC
1101 ACTGGGTGGT AGTCGTTGGC CTGGTGGTCC GCGTAGTCGT CCGCGTGGTC
CGCGTCCGGA TGGTCCGCAG CCGAGTCTGA GCCTGGCAGA ACAGCATCTG
1201 GAAAGTCCTG TGCCGAGCGC ACCGGGTGCA CTGGCAGGCG GTCCGACACA
GGCAGCACCT GGTGTTCGTG GTGAAGAAGA ACAGTGGGCA CGCGAAATTG
1301 GTGCACAGCT GCGTCGTATG GCAGATGATC TGAATGCACA GTATGAACGT
CGTCGTCAAG AAGAACAGCA GCGTCATCGT CCGAGCCCGT GGCGTGTTCT
1401 GTATAATCTG ATTATGGGTC TGCTGCCGCT GCCTCGTGGT CATCGTGCAC
CGGAAATGGA ACCGAATAAA GATGAACTG

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding

sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E. coli* strains BL21 (DE3) from Novagen and BL21DE3pLysSRIL from Stratagene. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 14. The fusion protein of SEQ. No. 14

The protein of SEQ. No. 14 is a fusion protein having the length of 186 amino acids and the mass of 21.5 kDa, in which at the N-terminus of the sequence TRAIL 121-281 the 8-amino acid fragment of the protein SMAC/Diablo (SEQ. No. 39) is attached as an effector peptide, the polyarginine sequence consisting of seven Arg residues being additionally attached to the C-terminus of the effector peptide. Furthermore, between the polyarginine sequence and the sequence of TRAIL the protein contains sequences of cleavage sites recognized by protease uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 3 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 14 and SEQ. No. 80 as shown below.

Amino acid sequence: SEQ. No. 14

1 AVPIAQKPRR RRRRRRVVRP LGLAGRVAAH ITGTRGRSNT LSSPNSKNEK
51 ALGRKINSWE SSRSGHSFLS NLHLRNGELV IHEKGFYYIY SQTYFRFQEE
101 IKENTKNDKQ MVQYIYKYTS YPDPILLMKS ARNSCWSKDA EYGLYSIYQG
151 GIFELKENDR IFVSVTNEHL IDMDHEASFF GAFLVG

DNA sequence: SEQ. No. 80

1 GCAGTTCCGA TTGCACAGAA ACCGCGTCGT CGTCGTCGCC GTCGTCGTGT
TGTTCGTCCG CTGGGTCTGG CAGGTCGTGT TGCAGCACAT ATTACCGGCA
101 CCCGTGGTCG TAGCAATACC CTGAGCAGCC CGAATAGCAA AAATGAAAAA
GCCCTGGGTC GCAAAATCAA TAGCTGGGAA AGCAGCCGTA GCGGTCATAG
201 CTTTCTGAGC AATCTGCATC TGCGTAATGG TGAACTGGTG ATTCATGAAA
AAGGCTTTTA CTATATCTAT AGCCAGACCT ACTTCCGCTT TCAGGAAGAA

301 ATTAAAGAAA ATACCAAAAA TGATAAACAA ATGGTGCAGT ATATCTATAA ATATACCAGC TATCCGGATC CGATTCTGCT GATGAAAAGC GCACGTAATA

401 GCTGTTGGAG CAAAGATGCA GAATATGGCC TGTATAGCAT TTATCAGGGT GGCATTTTTG AACTGAAAGA AAATGATCGC ATTTTTGTGA GCGTGACCAA

501 TGAACATCTG ATTGATATGG ATCATGAAGC CAGCTTTTTT GGTGCATTTC TGGTGGGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E.coli* strains BL21 (DE3) or Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 15. The fusion protein of SEQ. No. 15

The protein of SEQ. No. 15 is a fusion protein having the length of 191 amino acids and the mass of 22.2 kDa, in which at the N-terminus of the sequence TRAIL 121-281 buforin IIb (SEQ. No. 40) is attached as an effector peptide. Furthermore, between the effector peptide and the sequence of TRAIL the protein contains sequences of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 3 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 15 and SEQ. No. 81 as shown below.

Amino acid sequence: SEQ. No. 15

1 RAGLQFPVGR LLRRLLRRLL RVVRPLGLAG RVAAHITGTR GRSNTLSSPN

51 SKNEKALGRK INSWESSRSG HSFLSNLHLR NGELVIHEKG FYYIYSQTYF

101 RFQEEIKENT KNDKQMVQYI YKYTSYPDPI LLMKSARNSC WSKDAEYGLY

151 SIYQGGIFEL KENDRIFVSV TNEHLIDMDH EASFFGAFLV G

DNA sequence: SEQ. No. 81

1 CGTGCAGGTC TGCAGTTTCC GGTTGGACGT CTGTTACGTC GCCTGCTGCG
TCGTCTGCTG CGCGTTGTTC GTCCGCTGGG TCTGGCAGGT CGTGTTGCAG
101 CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG CAGCCCGAAT
AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATCAATAGCT GGGAAAGCAG
201 CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT AATGGTGAAC
TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA GACCTATTTT
301 CGCTTTCAAG AAGAGATTAA AGAAAATACC AAAAATGATA AACAAATGGT
GCAGTACATT TACAAATATA CCAGCTATCC GGACCCGATT CTGCTGATGA
401 AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA TGGTCTGTAT
AGCATTTATC AGGGTGGCAT CTTTGAGCTG AAAGAAAATG ATCGCATCTT
501 TGTTAGCGTG ACCAACGAAC ATCTGATCGA TATGGATCAT GAAGCCAGCT
TTTTTGGTGC ATTTCTGGTG GGTCTGGTTC CGCGTGGTAG CGGTAGCAGC
601 CATCATCATC ATCACCATAG CAGCGGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 16. The fusion protein of SEQ. No. 16

The protein of SEQ. No. 16 is a fusion protein having the length of 279 amino acids and mass of 31.7 kDa, in which at the C-terminus of the TRAIL 121-281 sequence protein onconase (SEQ. No. 41) is attached as an effector peptide. Between the sequence of TRAIL and the sequence of the effector peptide there is a sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52), additionally separated from the sequence of TRAIL by the flexible glycine-serine linker GGGS (SEQ. No. 58).

Structure of the fusion protein is shown schematically in Fig. 4 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 16 and SEQ. No. 82 as shown below.

Amino acid sequence: SEQ. No. 16

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGSGPLGLA GRVVRQDWLT FQKKHITNTR DVDCDNIMST
201 NLFHCKDKNT FIYSRPEPVK AICKGIIASK NVLTTSEFYL SDCNVTSRPC
251 KYKLKKSTNK FCVTCENQAP VHFVGVGSC

DNA sequence: SEQ. No. 82

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAATACC AAAAATGATA
AGCAGATGGT GCAGTATATC TATAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTA GCGGTCCGCT
501 GGGTCTGGCA GGTCGTGTTG TTCGTCAGGA TTGGCTGACC TTTCAGAAAA
AACATATTAC CAATACCCGT GATGTGGATT GCGATAATAT TATGAGCACC
601 AACCTGTTTC ATTGCAAAGA TAAAAATACC TTTATTTATA GCCGTCCGGA
ACCGGTTAAA GCAATTTGTA AAGGTATTAT TGCCAGCAAA AATGTGCTGA
701 CCACGAGCGA ATTCTATCTG AGCGATTGTA ATGTTACCAG CCGTCCGTGT
AAATATAAAC TGAAAAAAAG CACCAATAAA TTTTGCGTGA CCTGCGAAAA
801 TCAGGCACCG GTTCATTTTG TTGGTGTTGG TAGCTGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 17. The fusion protein of SEQ. No. 17

The protein of SEQ. No. 17 is a fusion protein having the length of 274 amino acids and the mass of 31 kDa, in which at the C-terminus of the TRAIL 121-281 sequence protein onconase (SEQ. No. 41) is attached as an effector peptide, the sequence of the effector peptide being additionally separated from the sequence of TRAIL by the flexible glycine-serine linker, GGGGSGGGGS (SEQ. No. 64).

Structure of the fusion protein is shown schematically in Fig. 4 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 17 and SEQ. No. 83 as shown below.

Amino acid sequence: SEQ. No. 17

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGGSGGGGS QDWLTFQKKH ITNTRDVDCD NIMSTNLFHC
201 KDKNTFIYSR PEPVKAICKG IIASKNVLTT SEFYLSDCNV TSRPCKYKLK
251 KSTNKFCVTC ENQAPVHFVG VGSC

DNA sequence: SEQ. No. 83

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAATACC AAAAATGATA
AGCAGATGGT GCAGTATATC TATAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCGGTGG
501 TGGTGGCAGC CAGGATTGGC TGACCTTTCA GAAAAAACAT ATTACCAATA
CCCGTGATGT GGATTGCGAT AATATTATGA GCACCAACCT GTTTCATTGC
601 AAAGATAAAA ATACCTTTAT TTATAGCCGT CCGGAACCGG TTAAAGCAAT
TTGTAAAGGT ATTATTGCCA GCAAAAATGT GCTGACCACG AGCGAATTCT

701 ATCTGAGCGA TTGTAATGTT ACCAGCCGTC CGTGTAAATA TAAACTGAAA
AAAAGCACCA ATAAATTTTG CGTGACCTGC GAAAATCAGG CACCGGTTCA
801 TTTTGTTGGT GTTGGTAGCT GT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 18. The fusion protein of SEQ. No. 18

The protein of SEQ. No. 18 is a fusion protein having the length of 197 amino acids and the mass of 23.2 kDa, in which at the N-terminus of the TRAIL 121-281 sequence a 20-amino acid peptide containing the N-terminal domain of the protein p14ARF (SEQ. No. 42) is attached as an effector peptide, the polyarginine sequence consisting of six Arg residues being additionally attached at the C-terminus of the effector peptide. Furthermore, between the polyarginine sequence and the sequence of TRAIL there is a sequence of protease cleavage sites uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 4 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 18 and SEQ. No. 84 as shown below.

Amino acid sequence: SEQ. No. 18

1 VRRFLVTLRI RRACGPPRVR RRRRRRRVVR PLGLAGRVAA HITGTRGRSN
51 TLSSPNSKNE KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI
101 YSQTYFRFQE EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD
151 AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG

DNA sequence: SEQ. No. 84

1 GTTCGTCGTT TTCTGGTTAC CCTGCGTATT CGTCGTGCAT GTGGTCCTCC
GCGTGTGCGT CGTCGTCGTC GCCGTCGTCG TGTTGTTCGT CCTCTGGGTC

101 TGGCAGGTCG CGTTGCAGCA CATATTACCG GCACCCGTGG TCGTAGCAAT
ACCCTGAGCA GCCCGAATAG CAAAAATGAA AAAGCCCTGG GTCGCAAAAT
201 TAATAGCTGG GAAAGCAGCC GTAGCGGTCA TAGCTTTCTG AGCAATCTGC
ATCTGCGTAA TGGTGAACTG GTGATTCATG AAAAAGGCTT TTATTATATT
301 TATAGCCAGA CCTATTTTCG CTTTCAGGAA GAAATTAAAG AAAATACCAA
AAATGATAAA CAAATGGTGC AGTATATCTA TAAATATACC AGCTATCCGG
401 ATCCGATTCT GCTGATGAAA AGCGCACGTA ATAGCTGTTG GAGCAAAGAT
GCAGAATATG GCCTGTATAG CATTTATCAG GGTGGCATTT TTGAACTGAA
501 AGAAAATGAT CGCATTTTTG TGAGCGTGAC CAATGAACAT CTGATTGATA
TGGATCATGA AGCCAGCTTT TTTGGTGCAT TTCTGGTTGG T

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 19. The fusion protein of SEQ. No. 19

The protein of SEQ. No. 19 is a fusion protein having the length of 189 amino acids and the mass of 22.3 kDa, in which at the N-terminus of the TRAIL 121-281 sequence the 11-amino acid peptide binding to Mdm2 (SEQ. No. 43) is attached as an effector peptide, the polyarginine sequence consisting of seven Arg residues being additionally attached at the C-terminus of the effector peptide. Furthermore, between the polyarginine sequence and the sequence of TRAIL there are sequences of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 4 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 19 and SEQ. No. 85 as shown below.

Amino acid sequence: SEQ. No. 19

1 PRFMDTWEGL NRRRRRRRRV VRPLGLAGRV AAHITGTRGR SNTLSSPNSK
51 NEKALGRKIN SWESSRSGHS FLSNLHLRNG ELVIHEKGFY YIYSQTYFRF

101 QEEIKENTKN DKQMVQYIYK YTSYPDPILL MKSARNSCWS KDAEYGLYSI
151 YQGGIFELKE NDRIFVSVTN EHLIDMDHEA SFFGAFLVG

DNA sequence: SEQ. No. 85

1 CCTCGTTTTA TGGATACCTG GGAAGGTCTG AATCGCCGTC GGCGTCGTCG
GCGTCGTGTT GTTCGTCCGC TGGGTCTGGC AGGTCGTGTT GCAGCACATA
101 TTACCGGCAC CCGTGGTCGT AGCAATACCC TGAGCAGCCC GAATAGCAAA
AATGAAAAAG CACTGGGTCG CAAAATTAAT AGCTGGGAAA GCAGCCGTAG
201 CGGTCATAGC TTTCTGAGCA ATCTGCATCT GCGTAATGGT GAACTGGTGA
TTCATGAAAA AGGCTTTTAT TATATTTATA GCCAGACCTA TTTTCGCTTT
301 CAGGAAGAAA TTAAAGAAAA TACCAAAAAT GATAAACAAA TGGTGCAGTA
CATTTACAAA TATACCAGCT ATCCGGATCC GATTCTGCTG ATGAAAAGCG
401 CACGTAATAG CTGTTGGAGC AAAGATGCAG AATATGGTCT GTATAGCATT
TATCAGGGTG GCATTTTTGA ACTGAAAGAA AATGATCGCA TTTTTGTGAG
501 CGTGACCAAT GAACATCTGA TTGATATGGA TCATGAAGCC AGCTTTTTTG
GTGCATTTCT GGTTGGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E.coli* strains BL21 (DE3) or Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 20. The fusion protein of SEQ. No. 20

The protein of SEQ. No. 20 is a fusion protein having the length of 195 amino acids and the mass of 22.9 kDa, in which at the N-terminus of the TRAIL121-281 sequence the peptide derived from lunasin (SEQ. No. 44) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL there are, in given order, the polyarginine sequence consisting of seven Arg residues and sequences of cleavage sites for proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 4 and its amino acid sequence and the DNA encoding sequence comprising codons optimized

for expression in *E. coli* are, respectively, SEQ. No. 20 and SEQ. No. 86 as shown below.

Amino acid sequence: SEQ. No. 20

1 CEKHIMEKIQ GRGDDDDRRR RRRRRVVRPL GLAGRVAAHI TGTRGRSNTL
51 SSPNSKNEKA LGRKINSWES SRSGHSFLSN LHLRNGELVI HEKGFYYIYS
101 QTYFRFQEEI KENTKNDKQM VQYIYKYTSY PDPILLMKSA RNSCWSKDAE
151 YGLYSIYQGG IFELKENDRI FVSVTNEHLI DMDHEASFFG AFLVG

DNA sequence: SEQ. No. 86

1 TGTGAAAAAC ATATTATGGA AAAAATTCAG GGTCGCGGTG ATGATGATGA
TCGCCGTCGG CGTCGTCGGC GTCGTGTTGT TCGTCCGCTG GGTCTGGCAG
101 GTCGTGTTGC AGCACATATT ACCGGCACCC GTGGTCGTAG CAATACCCTG
AGCAGCCCGA ATAGCAAAAA TGAAAAAGCA CTGGGTCGCA AAATTAATAG
201 CTGGGAAAGC AGCCGTAGCG GTCATAGCTT TCTGAGCAAT CTGCATCTGC
GTAATGGTGA ACTGGTGATT CATGAAAAAG GCTTTTATTA TATTTATAGC
301 CAGACCTATT TTCGCTTTCA GGAAGAAATT AAAGAAAATA CCAAAAATGA
TAAACAAATG GTGCAGTACA TTTACAAATA TACCAGCTAT CCGGATCCGA
401 TTCTGCTGAT GAAAAGCGCA CGTAATAGCT GTTGGAGCAA AGATGCAGAA
TATGGTCTGT ATAGCATTTA TCAGGGTGGC ATTTTTGAAC TGAAAGAAAA
501 TGATCGCATT TTTGTGAGCG TGACCAATGA ACATCTGATT GATATGGATC
ATGAAGCCAG CTTTTTTGGT GCATTTCTGG TTGGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E.coli* strains BL21 (DE3) or Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 21. The fusion protein of SEQ. No. 21

The protein of SEQ. No. 21 is a fusion protein having the length of 218 amino acids and the mass of 25.5 kDa, in which at the N-terminus of the TRAIL 121-281 sequence there is attached as an effector peptide a fragment of the 8-

amino acid protein Smac/Diablo (SEQ. No. 39) with attached to its C-terminus polyarginine sequence consisting of seven Arg residues. Furthermore, to the C-terminus of the TRAIL 121-281 sequence there is attached as a second effector peptide the peptide containing the BH3 domain of Bik protein (SEQ. No. 45), the second effector peptide having attached at its N-terminus polyarginine sequence consisting of seven Arg residues. Between the sequence of TRAIL and both effector peptides with attached polyarginine sequences there are sequences of cleavage sites recognized by metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Fig. 4 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 21 and SEQ. No. 87 as shown below.

Amino acid sequence: SEQ. No. 21

1 AVPIAQKPRR RRRRRRVVRP LGLAGRVAAH ITGTRGRSNT LSSPNSKNEK
51 ALGRKINSWE SSRSGHSFLS NLHLRNGELV IHEKGFYYIY SQTYFRFQEE
101 IKENTKNDKQ MVQYIYKYTS YPDPILLMKS ARNSCWSKDA EYGLYSIYQG
151 GIFELKENDR IFVSVTNEHL IDMDHEASFF GAFLVGPLGL AGRVVRRRRR
201 RRRLALRLAC IGDEMDVS

DNA sequence: SEQ. No. 87

1 GCAGTTCCGA TTGCACAGAA ACCGCGTCGT CGTCGTCGCC GTCGTCGTGT
TGTTCGTCCT CTGGGTCTGG CAGGTCGCGT TGCAGCACAT ATTACCGGCA
101 CCCGTGGTCG TAGCAATACC CTGAGCAGCC CGAATAGCAA AAATGAAAAA
GCCCTGGGTC GCAAAATTAA TAGCTGGGAA AGCAGCCGTA GCGGTCATAG
201 CTTTCTGAGC AATCTGCATC TGCGTAATGG TGAACTGGTG ATTCATGAAA
AAGGCTTTTA TTATATTTAT AGCCAGACCT ATTTTCGCTT TCAGGAAGAA
301 ATTAAAGAAA ATACCAAAAA TGATAAACAA ATGGTGCAGT ATATCTATAA
ATATACCAGC TATCCGGATC CGATTCTGCT GATGAAAAGC GCACGTAATA
401 GCTGTTGGAG CAAAGATGCA GAATATGGCC TGTATAGCAT TTATCAGGGT
GGCATTTTTG AACTGAAAGA AAATGATCGC ATTTTTGTGA GCGTGACCAA
501 TGAACATCTG ATTGATATGG ATCATGAAGC CAGCTTTTTT GGTGCATTTC
TGGTTGGTCC GCTGGGCCTG GCTGGCCGTG TGGTTCGCCG GCGCCGTCGC

601 CGTCGCCGCC TGGCACTGCG TCTGGCATGT ATTGGTGATG AAATGGATGT GAGC

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain Rosetta (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 22. The fusion protein of SEQ. No. 22)

The protein of SEQ. No. 22 is a fusion protein having the length of 199 amino acids and the mass of 22.3 kDa, in which at the C-terminus of the TRAIL 121-281 sequence there is attached as an effector peptide the synthetic peptide sequence consisting of Gly, Ala repetitions (SEQ. No. 46), having also attached to its C-terminus the polyarginine sequence consisting of eight Arg residues, the latter forming also the C-terminal part of the entire construct. Furthermore, between the effector peptide and the sequence of TRAIL there is a sequence of cleavage sites recognized by metalloprotease MMP (SEQ. No. 51) and urokinase uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Fig. 4 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 22 and SEQ. No. 88 as shown below.

Amino acid sequence: SEQ. No. 22

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GRVVRPLGLA GAGAGGGAGG AGAGGGAGGA GRRRRRRRR

DNA sequence: SEQ. No. 88

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT

101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAACACC AAAAATGATA
AACAAATGGT GCAGTATATT TACAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCGGTGG
501 TGGTCGTGTT GTTCGTCCGC TGGGTCTGGC TGGTGCCGGT GCCGGTGGTG
GTGCAGGCGG TGCTGGTGCG GGTGGCGGAG CCGGTGGTGC AGGTCGTCGT
601 CGTCGCCGTC GTCGGCGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E.coli* strains BL21 (DE3) or Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 23. The fusion protein of SEQ. No. 23

The protein of SEQ. No. 23 is a fusion protein having the length of 289 amino acids and the mass of 32.6 kDa, in which at the C-terminus of the 121-281 TRAIL sequence the C-terminal domain of the proteasome component S5a containing UIMs motifs (SEQ. No. 46) is attached as an effector peptide. Furthermore, between the effector peptide and the TRAIL sequence there is the sequence of furin cleavage site (SEQ. No. 53), additionally separated from the TRAIL sequence by flexible glycine-serine linker GGGSGG (SEQ. No. 61), and at the C-terminus of the effector peptide there is located the KEDL sequence directing to endoplasmic reticulum (SEQ. No. 56), the latter being the C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Fig. 5 and its amino acid sequence and the DNA encoding sequence comprising codons optimized

for expression in *E. coli* are, respectively, SEQ. No. 23 and SEQ. No. 89 as shown below.

Amino acid sequence: SEQ. No. 23

1 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
51 NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI
101 LLMKSARNSC WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH
151 EASFFGAFLV GGGGSGGRKK RMTISQQEFG RTGLPDLSSM TEEEQIAYAM
201 QMSLQGAEFG QAESADIDAS SAMDTSEPAK EEDDYDVMQD PEFLQSVLEN
251 LPGVDPNNEA IRNAMGSLAS QATKDGKKDK KEEDKKEDL

DNA sequence: SEQ. No. 89

1 CGTGTTGCAG CACATATTAC CGGCACCCGT GGTCGTAGCA ATACCCTGAG
CAGCCCGAAT AGCAAAAATG AAAAAGCACT GGGTCGCAAA ATTAATAGCT
101 GGGAAAGCAG CCGTAGCGGT CATAGCTTTC TGAGCAATCT GCATCTGCGT
AATGGTGAAC TGGTGATTCA TGAAAAAGGC TTTTATTATA TTTATAGCCA
201 GACCTATTTT CGCTTTCAAG AAGAAATTAA AGAAAACACC AAAAATGATA
AACAAATGGT GCAGTATATT TACAAATATA CCAGCTATCC GGATCCGATT
301 CTGCTGATGA AAAGCGCACG TAATAGCTGT TGGAGCAAAG ATGCAGAATA
TGGTCTGTAT AGCATTTATC AGGGTGGCAT TTTTGAACTG AAAGAAAATG
401 ATCGCATTTT TGTGAGCGTG ACCAATGAAC ATCTGATTGA TATGGATCAT
GAAGCCAGCT TTTTTGGTGC ATTTCTGGTT GGTGGTGGTG GTAGCGGTGG
501 TCGTAAAAAA CGTATGACCA TTAGCCAGCA AGAATTTGGT CGTACCGGTC
TGCCGGATCT GAGCAGCATG ACCGAAGAAG AACAAATTGC CTACGCAATG
601 CAGATGAGCC TGCAGGGTGC AGAATTTGGT CAGGCAGAAA GCGCAGATAT
TGATGCAAGC AGCGCAATGG ATACCAGCGA ACCGGCAAAA GAAGAAGACG
701 ATTACGACGT TATGCAGGAT CCGGAATTTC TGCAGAGCGT TCTGGAAAAT
CTGCCGGGTG TTGATCCGAA TAATGAAGCA ATTCGTAATG CAATGGGTAG
801 CCTGGCAAGC CAAGCAACCA AAGATGGCAA AAAAGATAAA AAAGAGGAAG
ACAAAAAAGA AGATCTG

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using

E.coli strains BL21 (DE3) or Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 24. The fusion protein of SEQ. No. 24 (comparative)

The protein of SEQ. No. 24 is a fusion protein having the length of 183 amino acids and the mass of 21 kDa, in which at the N-terminus of the 119-281 TRAIL sequence the decapeptide derived from the TNF ligand (SEQ. No. 48) is attached as an effector peptide. Furthermore, between the the sequence of the effector peptide and the sequence of TRAIL there are sequences of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 5 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 24 and SEQ. No. 90 as shown below.

Amino acid sequence: SEQ. No. 24

1 VANPQAEGQL RVVRPLGLAG PQRVAAHITG TRGRSNTLSS PNSKNEKALG
51 RKINSWESSR SGHSFLSNLH LRNGELVIHE KGFYYIYSQT YFRFQEEIKE
101 NTKNDKQMVQ YIYKYTSYPD PILLMKSARN SCWSKDAEYG LYSIYQGGIF
151 ELKENDRIFV SVTNEHLIDM DHEASFFGAF LVG

DNA sequence: SEQ. No. 90

1 GTTGCAAATC CGCAGGCAGA AGGTCAGCTG CGCGTTGTTC GTCCGCTGGG
TCTGGCAGGT CCGCAGCGTG TTGCAGCACA TATTACCGGC ACCCGTGGTC
101 GTAGCAATAC CCTGAGCAGC CCGAATAGCA AAAATGAAAA AGCCCTGGGT
CGTAAAATTA ATAGCTGGGA AAGCAGCCGT AGCGGTCATA GCTTTCTGAG
201 CAATCTGCAT CTGCGTAATG GCGAACTGGT GATTCATGAA AAAGGCTTTT
ATTATATTTA TAGCCAGACC TATTTTCGCT TTCAGGAAGA AATTAAAGAA
301 AATACCAAAA ATGATAAACA AATGGTGCAG TATATCTATA AATATACCAG
CTATCCGGAT CCGATTCTGC TGATGAAAAG CGCACGTAAT AGCTGTTGGA
401 GCAAAGATGC CGAATATGGT CTGTATAGCA TTTATCAGGG TGGCATTTTT
GAACTGAAAG AAAATGATCG CATTTTTGTG AGCGTGACCA ATGAACATCT
501 GATTGATATG GATCATGAAG CCAGCTTTTT TGGTGCATTT CTGGTTGGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain BL21 (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 25. The fusion protein of SEQ. No. 25 (comparative)

The protein of SEQ. No. 25 is a fusion protein having the length of 179 amino acids and the mass of 20.7 kDa, in which at the N-terminus of the TRAIL 119-281 sequence the 6-amino acid peptide derived from TNF (SEQ. No. 49) is attached as an effector peptide. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there are sequences of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 5 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 25 and SEQ. No. 91 as shown below.

Amino acid sequence: SEQ. No. 25

1 LANGVERVVR PLGLAGPQRV AAHITGTRGR SNTLSSPNSK NEKALGRKIN
51 SWESSRSGHS FLSNLHLRNG ELVIHEKGFY YIYSQTYFRF QEEIKENTKN
101 DKQMVQYIYK YTSYPDPILL MKSARNSCWS KDAEYGLYSI YQGGIFELKE
151 NDRIFVSVTN EHLIDMDHEA SFFGAFLVG

DNA sequence: SEQ. No. 91

1 CTGGCAAATG GTGTTGAACG TGTTGTTCGT CCGCTGGGTC TGGCAGGTCC
GCAGCGTGTT GCAGCACATA TTACCGGCAC CCGTGGTCGT AGCAATACCC
101 TGAGCAGCCC GAATAGCAAA AATGAAAAAG CCCTGGGTCG TAAAATTAAT
AGCTGGGAAA GCAGCCGTAG CGGTCATAGC TTTCTGAGCA ATCTGCATCT
201 GCGTAATGGC GAACTGGTGA TTCATGAAAA AGGCTTTTAT TATATTTATA
GCCAGACCTA TTTTCGCTTT CAGGAAGAAA TTAAAGAAAA TACCAAAAAT
301 GATAAACAAA TGGTGCAGTA TATCTATAAA TATACCAGCT ATCCGGATCC
GATTCTGCTG ATGAAAAGCG CACGTAATAG CTGTTGGAGC AAAGATGCCG

401 AATATGGTCT GTATAGCATT TATCAGGGTG GCATTTTTGA ACTGAAAGAA
AATGATCGCA TTTTTGTGAG CGTGACCAAT GAACATCTGA TTGATATGGA
501 TCATGAAGCC AGCTTTTTTG GTGCATTTCT GGTTGGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 26. The fusion protein of SEQ. No. 26 (comparative)

The protein of SEQ. No. 26 is a fusion protein having the length of 180 amino acids and the mass of 20.8 kDa, in which at the N-terminus of the TRAIL119-281 sequence there is attached as an effector peptide the 5-amino acid fragment of the TNF cytokine (SEQ. No 50) with additional one Cys residue at its both C-terminus and N-terminus. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there are sequences of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Fig. 5 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 26 and SEQ. No. 92 as shown below.

Amino acid sequence: SEQ. No. 26

1 CPSEGLCRVV RPLGLAGPQR VAAHITGTRG RSNTLSSPNS KNEKALGRKI
51 NSWESSRSGH SFLSNLHLRN GELVIHEKGF YYIYSQTYFR FQEEIKENTK
101 NDKQMVQYIY KYTSYPDPIL LMKSARNSCW SKDAEYGLYS IYQGGIFELK
151 ENDRIFVSVT NEHLIDMDHE ASFFGAFLVG

DNA sequence: SEQ. No. 92

1 TGTCCGAGCG AAGGTCTGTG TCGTGTTGTT CGTCCGCTGG GTCTGGCAGG
TCCGCAGCGT GTTGCAGCAC ATATTACCGG CACCCGTGGT CGTAGCAATA

101 CCCTGAGCAG CCCGAATAGC AAAAATGAAA AAGCCCTGGG TCGTAAAATT
AATAGCTGGG AAAGCAGCCG TAGCGGTCAT AGCTTTCTGA GCAATCTGCA
201 TCTGCGTAAT GGCGAACTGG TGATTCATGA AAAAGGCTTT TATTATATTT
ATAGCCAGAC CTATTTTCGC TTTCAGGAAG AAATTAAAGA AAATACCAAA
301 AATGATAAAC AAATGGTGCA GTATATCTAT AAATATACCA GCTATCCGGA
TCCGATTCTG CTGATGAAAA GCGCACGTAA TAGCTGTTGG AGCAAAGATG
401 CCGAATATGG TCTGTATAGC ATTTATCAGG GTGGCATTTT TGAACTGAAA
GAAAATGATC GCATTTTTGT GAGCGTGACC AATGAACATC TGATTGATAT
501 GGATCATGAA GCCAGCTTTT TTGGTGCATT TCTGGTTGGT

The amino acid sequence presented above was used as a template to generate its coding DNA sequence presented above. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E.coli* strain Tuner (DE3) from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 27. The fusion protein of SEQ. No. 93

The protein of SEQ. No. 93 is a fusion protein having the length of 459 amino acids and the mass of 50.4 kDa, in which at the C-terminus of the TRAIL95-281 sequence there is attached as an effector peptide the full length human RNAse A (SEQ. No 32) flanked at its C-terminus by the sequence directing to endoplasmic reticulum (KDEL) and at its N-terminus by the flexible glycine-serine linker (SEQ No. 175). Additionally, to stabilize its trimeric structure, the sequence of TRAIL has attached at its N-terminus the polycysteine linker (SEQ. No. 179) flanked at its N-terminus by glycine residue. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide and there are located in a given order the flexible glycine-serine linker (SEQ. No. 59), the linker for pegylation (SEQ. No. 170), the sequence of cleavage site recognized by furin (SEQ. No. 53), the flexible glycine-serine linker (SEQ No. 65) and the modified *Pseudomonas aeruginosa* translocation domain (helix F deletion) (SEQ. No 176).

Structure of the fusion protein is shown schematically in Fig. 6 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in E. coli are, respectively, SEQ. No. 93 and SEQ. No. 122.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using E. coli Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 28. The fusion protein of SEQ. No. 94

The protein of SEQ. No. 94 is a fusion protein having the length of 213 amino acids and the mass of 24.7 kDa, in which at the N-terminus of the TRAIL95-281 sequence the Nur77 derived peptide (SEQ. No 35) is attached as an effector peptide. The sequence of the effector peptide has attached at its N-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Between the sequence of the effector peptide and the sequence of TRAIL there is a sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Fig. 6 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in E. coli are, respectively, SEQ. No. 93 and SEQ. No. 122.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using E. coli Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 29. The fusion protein of SEQ. No. 95

The protein of SEQ. No. 95 is a fusion protein having the length of 204 amino acids and the mass of 23,1kDa, in which at the N-terminus of the sequence of

TRAIL116-281 the azurin derived peptide (SEQ. No 151) is attached as an effector peptide. Between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Figure 6 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 95 and SEQ. No. 124.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 30. The fusion protein of SEQ. No. 96

The protein of SEQ. No. 96 is a fusion protein having the length of 205 amino acids and the mass of 23,3 kDa, in which at the C-terminus of the sequence of TRAIL120-281 the azurin derived peptide (SEQ. No 151) is attached as an effector peptide. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is a sequence of cleavage site recognized by furin protease (SEQ. No. 172), additionally separated from the TRAIL sequence by the flexible glycine-serine linker GGGS (SEQ. No. 58). The C-terminus of the effector peptide is flanked by the sequence KEDL directing to endoplasmic reticulum (SEQ. No. 56), which forms the C-terminal part of the entire construct.

Structure of the fusion protein is shown schematically in Figure 6 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 96 and SEQ. No. 125.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion

protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 31. The fusion protein of SEQ. No. 97

The protein of SEQ. No. 97 is a fusion protein having the length of 207 amino acids and the mass of 23,1 kDa, in which at the C-terminus of the sequence of TRAIL120-281 the azurin derived peptide (SEQ. No 151) is attached as an effector peptide. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52), additionally separated from the sequence of TRAIL by the flexible glycine-serine linker GGGSGGG (SEQ. No. 62).

Structure of the fusion protein is shown schematically in Figure 6 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 97 and SEQ. No. 126.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 32. The fusion protein of SEQ. No. 98

The protein of SEQ. No. 97 is a fusion protein having the length of 327 amino acids and the mass of 36,2 kDa, in which at the C-terminus of the sequence of TRAIL120-281 the full length azurin peptide (SEQ. No 152) is attached as an effector peptide. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52), additionally separated from the sequence of TRAIL sequence by the flexible glycine-serine linker GGGSGGG (SEQ. No. 62).

Structure of the fusion protein is shown schematically in Figure 7 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 98 and SEQ. No. 127.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 33. The fusion protein of SEQ. No. 99

The protein of SEQ. No. 99 is a fusion protein having the length of 199 amino acids and the mass of 22,9 kDa, in which at the N-terminus of the sequence of TRAIL114-281 the Smac/DIABLO derived octameric peptide (SEQ. No 39) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 7 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 99 and SEQ. No. 128.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 34. The fusion protein of SEQ. No. 100

The protein of SEQ. No. 100 is a fusion protein having the length of 221 amino acids and the mass of 25,2 kDa, in which at the N-terminus of the sequence of TRAIL95-281 the Smac/DIABLO derived octameric peptide (SEQ. No 39) is attached as an effector peptide. The sequence of TRAIL has attached at its N-terminus the polycysteine linker (SEQ. No. 177) for stabilizing its trimeric structure. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 7 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 100 and SEQ. No. 129.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 35. The fusion protein of SEQ. No. 101

The protein of SEQ. No. 101 is a fusion protein having the length of 212 amino acids and the mass of 24,5 kDa, in which at the N-terminus of the sequence of TRAIL95-281 the Smac/DIABLO derived octameric peptide (SEQ. No 39) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 7 and its amino acid sequence and the DNA encoding sequence comprising codons

optimized for expression in *E. coli* are, respectively, SEQ. No. 101 and SEQ. No. 130.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 36. The fusion protein of SEQ. No. 102

The protein of SEQ. No. 102 is a fusion protein having the length of 212 amino acids and the mass of 24,5 kDa, in which at the N-terminus of the sequence of TRAIL121-281 the peptide designed from aPP protein and BH3 domain of Bax protein (SEQ. No 153) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 6 Arg residues. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there are located the sequences of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 7 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 102 and SEQ. No. 131.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 37. The fusion protein of SEQ. No. 103

The protein of SEQ. No. 103 is a fusion protein having the length of 247 amino acids and the mass of 28,1 kDa, in which at the N-terminus of the sequence of TRAIL95-281 the peptide designed from aPP protein and BH3 domain of Bax protein (SEQ. No 153) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 6 Arg residues. The sequence of TRAIL has attached at its N-terminus the polycysteine linker (SEQ. No. 177) to stabilize its trimeric structure. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 8 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 103 and SEQ. No. 132.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 38. The fusion protein of SEQ. No. 104

The protein of SEQ. No. 104 is a fusion protein having the length of 212 amino acids and the mass of 24,4 kDa, in which at the C-terminus of the sequence of TRAIL114-281 the peptide designed from aPP protein and BH3 domain of Bax protein (SEQ. No 153) is attached as an effector peptide. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Figure 8 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 104 and SEQ. No. 133.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 39. The fusion protein of SEQ. No. 105

The protein of SEQ. No. 105 is a fusion protein having the length of 221 amino acids and the mass of 24,8 kDa, in which at the N-terminus of the sequence of TRAIL120-281 Reticulon RTN1-C derived peptide (SEQ. No 155) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the nucleus localizing sequence (SEQ. No. 168). Additionally, to stabilize its trimeric structure, the sequence of TRAIL has attached at its N-terminus the polycysteine linker (SEQ. No. 179) flanked by two and three glycine residues, respectively at its N- and C-terminus. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 8 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 105 and SEQ. No. 134.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 40. The fusion protein of SEQ. No. 106

The protein of SEQ. No. 106 is a fusion protein having the length of 435 amino acids and the mass of 48 kDa, in which at the N-terminus of the sequence of

TRAIL119-281 the Reticulon RTN1-C derived peptide (SEQ. No 156) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 8 Arg residues. Additionally, to stabilize its trimeric structure, the sequence of TRAIL has attached at its N-terminus the polycysteine linker (SEQ. No. 178). Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51), this sequence of cleavage sites being flanked by a linker sequence GGSGG (SEQ. No. 60), respectively at the N- and C-terminus.

Structure of the fusion protein is shown schematically in Figure 8 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 106 and SEQ. No. 135.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 41. The fusion protein of SEQ. No. 107

The protein of SEQ. No. 107 is a fusion protein having the length of 580 amino acids and the mass of 65 kDa, in which at the C-terminus of the sequence of TRAIL121-281 constitutively active caspase-3 (single chain) (SEQ. No 157) is attached as an effector peptide. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the transporting domain derived from *Pseudomonas* (SEQ No. 176). The transporting domain and the sequence of the effector peptide are connected via flexible linker GGGSGGG (SEQ. No. 62). The transporting domain is separated from the sequence of TRAIL by the sequence of the cleavage site recognized by furin (SEQ. No. 53), this sequence of cleavage site being

flanked at its N- and C-terminus by two linker sequences GGGGS (SEQ. No. 59) and ASGG (SEQ. No. 65), respectively.

Structure of the fusion protein is shown schematically in Figure 8 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 107 and SEQ. No. 136.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 42. The fusion protein of SEQ. No. 108

The protein of SEQ. No. 108 is a fusion protein having the length of 247 amino acids and the mass of 28,5 kDa, in which at the N-terminus of the sequence of TRAIL119-281 SAC domain from Par-4 (SEQ. No 158) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Additionally, the sequence of TRAIL has attached at its N-terminus the flexible glycine-serine linker GGSGG (SEQ. No. 60). Furthermore, between the sequence of TRAIL and sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 173).

Structure of the fusion protein is shown schematically in Figure 9 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 108 and SEQ. No. 137.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A,

using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 43. The fusion protein of SEQ. No. 109

The protein of SEQ. No. 109 is a fusion protein having the length of 247 amino acids and the mass of 28,5 kDa, in which at the N-terminus of the sequence of TRAIL119-281 the SAC domain from Par-4 (SEQ. No 158) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 7 Arg residues, and at its N-terminus the NLS (Nuclear Localization Signal) sequence from Oct6 transcription factor (SEQ. No. 168). The sequence of TRAIL has attached at its N-terminus the flexible glycine-serine linker GGSGG (SEQ. No. 60). Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 173).

Structure of the fusion protein is shown schematically in Figure 9 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 109 and SEQ. No. 138.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 44. The fusion protein of SEQ. No. 110

The protein of SEQ. No. 110 is a fusion protein having the length of 270 amino acids and the mass of 30,8 kDa, in which at the C-terminus of the sequence of TRAIL95-281 Noxa protein (SEQ. No 159) is attached as an effector peptide. The sequence of the effector peptide has attached at its N-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Additionally, to stabilize its trimeric structure, the sequence of TRAIL has attached at its C-terminus the polycysteine linker (SEQ. No. 177), separated from the sequence

of TRAIL by the flexible glycine-serine linker GGSG (SEQ. No. 57). Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Figure 9 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 110 and SEQ. No. 139.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 45. The fusion protein of SEQ. No. 111

The protein of SEQ. No. 111 is a fusion protein having the length of 207 amino acids and the mass of 23,7 kDa, in which at the C-terminus of the sequence of TRAIL114-281 the MTD/CKP peptide derived from Noxa protein (SEQ. No 160) is attached as an effector peptide. The sequence of the effector peptide has attached at its N-terminus the poly-arginine transporting domain consisting of 7 Arg residues. To stabilize its trimeric structure, the sequence of TRAIL has attached at its C-terminus the polycysteine linker (SEQ. No. 177), this linker being separated from the sequence of TRAIL by the flexible glycine-serine linker GGSG (SEQ. No. 57). Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Figure 9 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 111 and SEQ. No. 140.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 46. The fusion protein of SEQ. No. 112

The protein of SEQ. No. 112 is a fusion protein having the length of 311 amino acids and the mass of 35 kDa, in which at the N-terminus of the sequence of TRAIL95-281 peptide onconase (SEQ. No 41) is attached as an effector peptide. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51), additionally separated from the sequence of TRAIL by two flexible glycine-serine linkers GGGGS (SEQ. No. 59).

Structure of the fusion protein is shown schematically in Figure 9 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 112 and SEQ. No. 141.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 47. The fusion protein of SEQ. No. 113

The protein of SEQ. No. 113 is a fusion protein having the length of 230 amino acids and the mass of 27 kDa, in which at the N-terminus of the sequence of TRAIL95-281 BH3 domain from PUMA protein (SEQ. No 37) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 9 Arg residues.

Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 10 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 113 and SEQ. No. 142.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 48. The fusion protein of SEQ. No. 114

The protein of SEQ. No. 114 is a fusion protein having the length of 225 amino acids and the mass of 25,7 kDa, in which at the C-terminus of the sequence of TRAIL95-281 the short peptide derived from Bid protein (SEQ. No 31) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the transporting domain KPRRPY (SEQ. No. 167). To stabilize its trimeric structure, the sequence of TRAIL has attached at its C-terminus the polycysteine linker (SEQ. No. 177). Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52).

Structure of the fusion protein is shown schematically in Figure 10 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 114 and SEQ. No. 143.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described

above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 49. The fusion protein of SEQ. No. 115

The protein of SEQ. No. 115 is a fusion protein having the length of 234 amino acids and the mass of 26,7 kDa, in which at the C-terminus of the sequence of TRAIL95-281 the short hybrid peptide Antp-TPR (SEQ. No 161) is attached as an effector peptide. Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52), additionally separated from the TRAIL sequence by polycysteine linker (SEQ. No. 177) to stabilize its trimeric structure, followed by two glycine residues.

Structure of the fusion protein is shown schematically in Figure 10 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 115 and SEQ. No. 144.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 50. The fusion protein of SEQ. No. 116

The protein of SEQ. No. 116 is a fusion protein having the length of 216 amino acids and the mass of 24,3 kDa, in which at the N-terminus of the sequence of TRAIL120-281 peptide inhibitor of the SH2 domain of Stat3 protein (SEQ. No 162) is attached as an effector peptide. Additionally, to stabilize its trimeric structure, at the N-terminus of the sequence of TRAIL there is attached the polycysteine linker (SEQ. No. 179), the linker being flanked at its N- and C-terminus by three glycine residues and GSG motif, respectively. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL

there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 10 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 116 and SEQ. No. 145.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 51. The fusion protein of SEQ. No. 117

The protein of SEQ. No. 117 is a fusion protein having the length of 194 amino acids and the mass of 22,8kDa, in which at the N-terminus of the sequence of TRAIL121-281 the peptide derived from BH3 domain of Bak protein (SEQ. No 163) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51).

Structure of the fusion protein is shown schematically in Figure 10 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 117 and SEQ. No. 146.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A,

using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 52. The fusion protein of SEQ. No. 118

The protein of SEQ. No. 118 is a fusion protein having the length of 257 amino acids and the mass of 30kDa, in which at the N-terminus of the sequence of TRAIL121-281 the peptide derived from BH3 domain of Bad protein (SEQ. No 164) is attached as an effector peptide. The sequence of the effector peptide has attached at its C-terminus the poly-arginine transporting domain consisting of 8 Arg residues. Between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51). At the C-terminus of the sequence of TRAIL121-281 there is the flexible linker GGSHG (SEQ. No. 182), followed by the sequence of the cleavage site recognized by thrombin protease (SEQ. No. 174) and, as a C-terminal part of the whole construct, the sequence of TRAIL95-121.

Structure of the fusion protein is shown schematically in Figure 11 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 118 and SEQ. No. 147.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 53. The fusion protein of SEQ. No. 119

The protein of SEQ. No. 119 is a fusion protein having the length of 236 amino acids and the mass of 27,5 kDa, in which at the C-terminus of the sequence of TRAIL95-281 the peptide derived from BH3 domain of Bad protein (SEQ. No 164) is attached as an effector peptide. The sequence of the effector peptide has attached at its N-terminus the poly-arginine transporting domain consisting of 7 Arg residues. Furthermore, between the sequence of TRAIL and

the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52), C-terminus of the sequence of TRAIL95-281 being additionally separated from the sequence of cleavage sites by the linker consisting of GGS residues.

Structure of the fusion protein is shown schematically in Figure 11 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 119 and SEQ. No. 148.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 54. The fusion protein of SEQ. No. 120

The protein of SEQ. No. 120 is a fusion protein having the length of 216 amino acids and the mass of 24,7 kDa, in which at the C-terminus of the sequence of TRAIL121-281 the ATAP peptide from Bfl1 protein (SEQ. No 165) is attached as an effector peptide. The sequence of the effector peptide has attached at its N-terminus the membrane transporting domain KPRRPYR (SEQ. No. 181). Furthermore, between the sequence of TRAIL and the sequence of the effector peptide there is located the sequence of cleavage sites recognized by proteases MMP (SEQ. No. 51) and uPA (SEQ. No. 52), additionally separated from the sequence of TRAIL by flexible glycine-serine linker GGGGSGGGG (SEQ. No. 180).

Structure of the fusion protein is shown schematically in Figure 11 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 120 and SEQ. No. 149.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion

protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 55. The fusion protein of SEQ. No. 121

The protein of SEQ. No. 120 is a fusion protein having the length of 237 amino acids and the mass of 27 kDa, in which at the N-terminus of the sequence of TRAIL121-281 the ATAP peptide from Bfl1 protein (SEQ. No 165) is attached as an effector peptide. The sequence of the effector peptide has attached at its N-terminus the mitochondrial targeting sequence (SEQ. No. 166). Furthermore, between the sequence of the effector peptide and the sequence of TRAIL there is located the sequence of cleavage sites recognized by proteases uPA (SEQ. No. 52) and MMP (SEQ. No. 51), additionally separated from the sequence of TRAIL by the flexible glycine-serine linker GGSGG (SEQ. No. 60).

Structure of the fusion protein is shown schematically in Figure 11 and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in *E. coli* are, respectively, SEQ. No. 121 and SEQ. No. 150.

The amino acid sequence of the structure described above was used as a template to generate its coding DNA sequence. A plasmid containing the coding sequence of DNA was generated and overexpression of the fusion protein was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli* Tuner (DE3) strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Examination of the anti-tumour activity of the fusion proteins

Examination of the anti-tumour activity of the fusion proteins was carried out *in vitro* in a cytotoxicity assay on tumour cell lines and *in vivo* in mice. For comparison purposes, the hTRAIL114-281 protein (hereinafter also designated as simply TRAIL) was used.

1. Tests on cell lines *in vitro*

Cell Lines

The cells of human colorectal cancer Colo205 (ATCC # CCL-222), small cell lung cancer A549 (ATCC # CCL-185), pancreatic cancer BxPC3 (ATCC # CRL-1687), prostate cancer DU145 (ATCC # HTB-81) and PC3 (ATCC # CRL-1435), and human large cell lung cancer NCI-H460-Luc2 (Caliper # 124316) were maintained in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum. Human ovarian cancer cells OVCAR-3 (ATCC # HTB-161) were maintained in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 20% fetal calf serum and 0.01 mg / ml insulin. Bladder cancer cells UM-UC-3 (ATCC # CRL-1749), lung cancer cells SK-MES-1 (ATCC # HTB-58), breast cancer cells MCF-7 (ATCC # HTB-22), HT1080 connective tissue cancer cells (ATCC # CCL-121), liver hepatoma HepG2 cells (ATCC # HB-8065) were maintained in MEM culture medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA). Connective tissue tumour cells HT1080 were maintained also during the experiment in the conditioned medium, harvested from 2-day normal culture of these cells. The cells of human colorectal cancer HCT-116 (ATCC # CCL-247) and HT-29 (HTB-38), ovarian cancer SK-OV-3 (ATCC # HTB-77), uterus cancer MES-SA (ATCC # CRL- 1976) and its clone resistant to doxorubicin MES-SA/Dx5 (ATCC # CRL-1977) were maintained in McCoy's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum. Bladder cancer cells SW780 (ATCC # CRL-2169), breast cancer cells MDA-MB-231 (ATCC # HTB-26) and human pancreatic carcinoma epithelial-like cell line PANC-1, CLS (Cell Lines Service # 300228 were maintained in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum. HUVEC cells from the umbilical vein (ATCC # CRL-1730) were maintained in M199 medium (Hyclone, Logan, UT, USA) supplemented with 20% fetal calf serum, growth factors 0.02 mg/ml ECGS (Sigma), 0.1 mg/ml heparin (Sigma), these cells were grown on a medium coated with 0.1% gelatin. MCF10A breast cells (ATCC # CRL-10317) were maintained in DMEM: F12 (1:1) (Sigma, USA) supplemented with 5% horse serum, 0.5 mg/ml hydrocortisone, 10 µg/ml insulin, 20 ng/ml growth factor EGF (all Sigma, USA). All media were additionally supplemented with 2mM L-glutamine and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin (Hyclone, Logan, UT, USA)). Cells were maintained at 37°C in 5% CO_2/air in

the case of growth media RPMI, MEM, McCoy and DMEM: F12, and in 10% CO_2/air in the case of DMEM. Cells were routinely checked for the presence of Mycoplasma by PCR technique using the kit Venor®GeM Mycoplasma PCR Detection Kit (Minerva Biolabs, Berlin, Germany).

MTT cytotoxicity test

MTT assay is a colorimetric assay used to measure cell proliferation, viability and cytotoxicity. It consists in decomposition of a yellow tetrazolium salt MTT (4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) to the water-insoluble purple dye formazan by the enzyme succinate-tetrazolium reductase present in the mitochondria. MTT reduction occurs only in living cells. Data analysis consists in determining the IC_{50} concentration of the protein (in ng/ml), at which the 50% reduction in the number of cells occurs in the population treated compared with the control cells. Results were analyzed using GraphPad Prism 5.0.

The test was performed according to the literature descriptions (Celis, JE, (1998). Cell Biology, a Laboratory Handbook, second edition, Academic Press, San Diego; Yang, Y., Koh, LW, Tsai, JH., (2004); Involvment of viral and chemical factors with oral cancer in Taiwan, Jpn J Clin Oncol, 34 (4), 176-183).

Cell culture medium was diluted to a defined density (10^4 - 10^5 cells per 100 µl). Then 100 µl of appropriately diluted cell suspension was applied to a 96-well plate in triplicates. Thus prepared cells were incubated for 24 h at 37°C in 5% or 10% CO_2, depending on the medium used, then to the cells (in 100 µl of medium) was added further 100 µl of the medium containing various concentrations of tested proteins. Cells were incubated with tested proteins over the next 72 hours which is equivalent to 3-4 times of a cell division, after which the medium with the test protein was added with 20 ml of working solution of MTT [5 mg/ml] and incubated for 3 h at 37°C in 5% CO_2. Then the medium with a solution of MTT was removed, and formazan crystals were dissolved by adding 100 µl of DMSO. After mixing, the absorbance was measured at 570 nm (reference filter 690 nm).

The results of *in vitro* cytotoxicity tests are summarized in Tables 1, 1a, 1b and Table 2 as IC_{50} values (ng/ml), which correspond to a protein concentration at which the cytotoxic effect of fusion proteins is observed at

the level of 50% with respect to control cells incubated with solvent only. Each experiment represents the average value of at least two independent experiments conducted in triplicates. As a criterion of lack of activity of protein preparations the IC_{50} limit of 2000 ng/ml was adopted. Fusion proteins with an IC_{50} value above 2000 were considered inactive.

Cells for this test were selected so as to include the tumour cell lines naturally resistant to TRAIL protein (the criterion of natural resistance to TRAIL: IC_{50} for TRAIL protein > 2000), tumour cell lines sensitive to TRAIL protein and resistant to doxorubicin line MES-SA/DX5 as a cancer line resistant to conventional anticancer medicaments.

Undifferentiated HUVEC cell line was used as a healthy control cell line for assessment of the impact/toxicity of the fusion proteins on non-cancer cells.

The results obtained confirm the possibility of overcoming the resistance of the cell lines to TRAIL by administration of certain fusion proteins of the invention to cells naturally resistant to TRAIL. When administering fusion proteins of the invention into the cells sensitive to TRAIL, in some cases a clear and strong potentiation of the potency of TRAIL action was observed, manifesting in reduced IC_{50} values of the fusion protein compared with the IC_{50} for the TRAIL alone. Furthermore, cytotoxic activity of the fusion protein of the invention was obtained on the cells resistant to classical anti-cancer medicament doxorubicin, in some cases being stronger than activity of TRAIL.

The IC_{50} values above 2000 obtained for the non-cancer cell lines show the absence of toxic effects associated with the use of proteins of the invention for healthy cells, which indicates potential low systemic toxicity of the protein.

Table 1. Cytotoxic activity of the fusion proteins of the invention and comparative proteins

Protein	Continuous incubation of preparations with cells over 72h (test MTT, ng/ml)											
	MES-SA		MES-SA/Dx5		HCT116		SK-MES-1		A549		MCF10A	
	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD
TRAIL 114-281	>2000		32.2	2.40	173	31.3	12.2	2.33	>2000		>2000	
Ex. 1	6.98	1.01	7.05	0.63	39.2	11.00	2.79	0.70	>2000		386	52.5
Ex. 2	3.19	0.41	2.62	1.61	35.1	23.70	6.43	1.22	>2000		>2000	
Ex. 5	646	166.9	378	94.3	757	446.3	1114	108.2	719	91.7	912	2.4
Ex. 9	>2000		1720	312.7	>2000		791.9	95.8	>2000		>2000	
Ex. 14	8.99	8.73	0.53	0,265	7.73	5.45	0.45	0.091	>2000		>2000	
Ex. 18	312	110.6	326	56.1	937	144.6	184	30.5	>2000		>2000	
Ex.19	24.9	21.2	19.9	1.98	23.9	2.31	87.6	32.4	87.2	45.8	83.1	19.33
Ex.20	259	60.0	172	20.9	223	110.4	123	25.6	296	3.4	282	39.9
Ex.24 (not of the invention)	>2000		1760	367.7	85.6	19.96	36.8	7.44	>2000		>2000	
Ex.25 (not of the invention)	>2000		157	40.0	991	119.0	117.4	4.24	>2000		>2000	
Ex.26 (not of the invention)	>2000		>2000		1895	70.0	245	19.2	>2000		>2000	

Table 1a. Cytotoxic activity of the fusion proteins of the invention

Protein	Continuous incubation of preparations with cells over 72h (test MTT, ng/ml)											
	A549		HCT116		MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1	
	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD
TRAIL 95-281	10000		7558		10000		10000		29.15	12.66	33.60	
Ex. 16	2632.50	219.91	132.65	37.69	1890.00	894.03	65.63	4.41	32.24	7.86	20.31	2632.50
Ex. 23	10000		223.55	105.29	10000		1280.00	304.06	292.50	86.97	82.46	1,48
Ex. 42	31.70	11.74	15.32	12.85	53.33	12.40	5.18	2.20	0.40	0.10	2.53	2.15
Ex. 36	142.05	32.46	5.66	2.26	79.16	3.33	2392.50	2.12	0.58	0.10	3.95	
Ex.3	3.10		9.43		4573		57.14		10.67		6.83	
Ex. 35	889.55	276.41	14.10		1273.50		57.14		1.18	0.82	3.93	0.32
Ex. 51 TRP15	307.95	72.05	1.29	1.41	4.97	1.50	0.64	0.34	0.08	0.11	0.62	

Table 1b. Cytotoxic activity of the fusion proteins of the invention

Protein	Continuous incubation of preparations with cells over 72h (test MTT, ng/ml)									
	HT29		H460		PLC/PRF/5		HepG2		PANC1	
	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD
TRAIL 95-281	10000		9000		10000		10000		10000	
Ex.3	108		2.852	3.10						
Ex. 35 .	3289.50		900.15							
Exp. 51					9.66		10.49		22.91	

2. Analysis of cytotoxic activity of selected protein preparations against extended panel of tumour cell lines

Table 2 presents the results of cytotoxic activity *in vitro* for selected fusion proteins of the invention against a broad panel of tumour cells from different organs, corresponding to the broad range of most common cancers. Obtained IC_{50} values confirm high cytotoxic activity of fusion proteins and thus their potential usefulness in the treatment of cancer.

Table 2. Analysis of cytotoxic activity of selected protein preparations against broad panel of tumour cell lines

Cell line	Continuous incubation of preparations with cells over 72h (test MTT, ng/ml)							
	TRAIL 114-281		Ex.1		Ex.2		Ex.14	
	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD	IC_{50}	±SD
UM-UC-3	39.05	5.70	0.44	0.44	0.55	0.21	1.72	0.97
HCT116	109.1	24.45	39.20	11	11.13	0.69	3.03	0.42
Colo205	7.32	1.46	2.26	0.30	1.06	0.06	0.47	0.09
SW780	25.53	5.10	3.86	1.39	0.99	0.15	0.18	0.03
A549	>2000		>2000		>2000		485.3	55.51
MDA-MB-231	81.18	3.78	11.66	2.06	23.80		7.89	2.81
MES-SA/Dx5	495.6	209.19	7.20	0.84	17.58	4.7	0.68	0.12
MES-SA	>2000		6.99	1.01	3.40		1.80	0.49
OVCAR-3	447.9	4.60	1.72	0.84	1.25	0.48	1.61	0.42
SK-MES-1	10.52	5.49	3.29	0.70	5.44	0.36	0.44	0.13
BxPC-3	13.20	1.56	3.89	1.74	1.24	0.54	1.55	0.48
DU145	>2000		10.76	1.63	137.8	120.3	5.89	2.3
HUVEC	>2000		>2000		>2000		>2000	

3. Antitumour effectiveness of fusion proteins *in vivo* on xenografts

Antitumour activity of protein preparations was tested in a mouse model of human colon cancer Colo205, human large cell lung cancer NCI-H460-Luc2, human lung cancer A549, and human pancreatic cancer PANC-1.

Cells

Colo205 cells (ATCC # CCL-222) were maintained in RPMI 1640 medium (Hyclone, Logan, UT, USA) mixed in the ratio of 1:1 with Opti-MEM ((Invitrogen, Cat.22600-134) supplemented with 10% fetal calf serum and 2 mM glutamine. On the day of mice grafting, the cells were detached from the support by washing the cells with trypsin (Invitrogen), then the cells were centrifuged at 1300 rpm, 4°C, 8 min., suspended in HBSS buffer (Hanks medium), counted and diluted to the concentration of $28.57x10^6$ cells/ml. Then to the cells Matrigel (BD Biocsciences, Cat.354 248) was added to the final cells concentration $25x10^6$ cells/ml.

H460-Luc2 cells were maintained in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal calf serum and 2 mM glutamine. On the day of mice grafting, the cells were detached from the support by washing the cells with trypsin (Invitrogen), then the cells were centrifuged at 1300 rpm, 4°C, 8 min., suspended in HBSS buffer (Hanks medium), counted and diluted to the concentration of $50x10^6$ cells/ml.

A549 cells were maintained in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal calf serum and 2 mM glutamine. On the day of mice grafting, the cells were detached from the support by washing the cells with trypsin (Invitrogen), then the cells were centrifuged at 1300 rpm, 4°C, 8 min., suspended in HBSS buffer (Hanks medium).

Human pancreatic cancer PANC-1 cells were maintained in DMEM medium (HyClone, Logan, UT, USA) supplemented with 10% fetal calf serum and 2 mM glutamine. On the day of mice grafting, the cells were detached from the support by washing the cells with trypsin (Invitrogen), then the cells were centrifuged at 1300 rpm, 4°C, 8 min., suspended in HBSS buffer (Hanks medium).

Mice

Examination of antitumor activity of proteins of the invention was conducted on 7-9 week-old NOD SCID mice obtained from Harlan UK Ltd., Shaws Farm, Bicester, UK. In the case of A549, NCI-H460-Luc2 and PANC-1 cells the examination of antitumor activity of the proteins of the invention was conducted on 4-5 week old Crl:SHO-Prkdc^{scid}Hr^{hr} mice obtained from Charles River Germany. Mice were kept under specific pathogen-free conditions with free access to food and demineralised water (*ad libitum*). All experiments on animals were carried in accordance with the guidelines: "Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education" issued by the New York Academy of Sciences' Ad Hoc Committee on Animal Research and were approved by the IV Local Ethics Committee on Animal Experimentation in Warsaw (No. 71/2009).

The course and evaluation of the experiment

On day 0 mice were grafted subcutaneously (sc) in the right side with $5x10^6$ of Colo205 cells suspended in 0.15 ml HBSS buffer and 0.05 ml of Matrigel by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumours reached the size of ~ 90-140 mm^3 (day 11), mice were randomized to obtain the average size of tumours in the group of ~ 115 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention and TRAIL114-281 as a comparison. The preparations were administered intraperitoneally (*ip*) daily for ten days (qdx10) on days 11-20. When a therapeutic group reached the average tumour size of ~ 2000 mm^3, the mice were sacrificed through disruption of the spinal cord. The control group received TRAIL114-281.

In the case of H460, on day 0 mice were grafted subcutaneously (sc) in the right side with $5x10^6$ of NCI-H460-Luc2 cells suspended in 0.1 ml HBSS buffer by means of a syringe with a needle 0.5 x25 mm (Bogmark). When tumours reached the size of ~ 100-120 mm^3 (day 11), mice were randomized and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention and TRAIL114-281 as a comparison. The preparations were administered intravenously (*i.v.*) 6 times daily every second day. On the 29th day of experiment the mice were

sacrificed through disruption of the spinal cord. The control group received TRAIL114-281.

In the case of A549, on day 0 mice were grafted subcutaneously (sc) in the right side with $7x10^6$ of A549 cells suspended in 0.1 ml of mixture HBSS buffer: Martigel in a ratio 3:1 by means of a syringe with a needle 0.5 x25 mm (Bogmark). When tumours reached the size of ~ 100-120 mm^3 (day 17), mice were randomized and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention and TRAIL114-281 as a comparison. The preparations were administered intravenously (*i.v.*) 6 times once daily every second day. In the 34^{th} day of experiment the mice were sacrificed through disruption of the spinal cord. The control group received TRAIL114-281.

In the case of PANC-1, on day 0 mice were grafted subcutaneously (sc) in the right side with 7x106 of PANC-1 cells suspended in 0.1 ml of mixture HBSS buffer: Martigel in a ratio 3:1 by means of a syringe with a needle 0.5 x25 mm (Bogmark). When tumours reached the size of ~ 95 mm3 (day 27), mice were randomized and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention and TRAIL114-281 as a comparison. The preparations were administered intravenously (i.v.) 6 times once daily every second day. In the 43th day of experiment the mice were sacrificed through disruption of the spinal cord. The control group received TRAIL114-281.

Tumour size was measured using an electronic calliper, tumour volume was calculated using the formula: $(a^2 \times b)/2$, where a = shorter diagonal of the tumour (mm) and b = longer diagonal of the tumour (mm). Inhibition of tumour growth was calculated using the formula:

TGI [%] (Tumour growth inhibition) = (WT/WC) x 100 - 100%

wherein WT refers to the average tumour volume in the treatment group, WC refers to the average tumour volume in the control group.

The experimental results are presented as a mean value ± standard deviation (SD). All calculations and graphs were prepared using the program GraphPad Prism 5.0.

The experimental results are shown in Fig. 12 and 13 as a diagram of changes of the tumour volume in mice SCID/NOD burdened with Colo205 colon cancer treated with fusion proteins of the invention and comparatively with TRAIL114-281. The results of experiments presented in the graphs in Figures 12 and 13 show that administration of the fusion proteins of the invention of Example 1 and Example 14 caused tumour Colo205 growth inhibition, with TGI respectively 39% and 32% relative to the control on 29th day of the experiment. For TRAIL114-281 used as the reference preparation, a slight inhibitory effect on tumour cell growth was obtained relative to the control, with TGI at the level of 9%. Thus, fusion proteins of the invention exert much stronger effect compared to TRAIL.

The tested fusion proteins did not cause significant side effects manifested by a decrease in body weight of mice (i.e. less than 10% of the baseline body weight). This shows low systemic toxicity of the protein.

The experimental results presented in Fig. 14 and 15 show a diagram of changes of the tumour volume in mice Crl:SHO-PrkdcscidHrhr burdened with NCI-H460 human large cell lung cancer treated with fusion proteins of the invention and comparatively with TRAIL114-281. It can be seen that by administering fusion proteins of the invention of Example 14 and Example 2 NCI-H460 tumour growth inhibition was obtained, with TGI respectively 82% and 81% relative to the control on 29th day of the experiment. For TRAIL114-281 used as the reference preparation, a slight inhibitory effect on tumour cell growth was obtained relative to the control, with TGI at the level of 75%. Thus, fusion proteins of the invention exert a much stronger effect against this cancer cells compared with TRAIL.

The tested fusion proteins did not cause significant side effects manifested by a decrease in body weight of mice (i.e. less than 10% of baseline body weight). This shows a low systemic toxicity of the protein.

The experimental results presented in Fig. 16 and 17 show a diagram of changes of the tumour volume in mice Crl:SHO-Prkdc^{scid}Hr^{hr} burdened with A549 human lung cancer treated with fusion proteins of the invention and comparatively with TRAIL114-281. It can be seen that by administering fusion proteins of the invention of Example 14 and Example 2 A549 tumour growth

inhibition was obtained, with TGI respectively 48% and 45,5% relative to the control on 29th day of the experiment. For TRAIL114-281 used as the reference preparation, a slight inhibitory effect on tumour cell growth was obtained relative to the control, with TGI at the level of 20,7%. Thus, fusion proteins of the invention exert a much stronger effect compared with TRAIL.

The experimental results presented in Fig. 18 and 19 show a diagram of changes of the tumour volume in mice Crl:SHO-PrkdcscidHrhr burdened with PANC-1 human pancreatic carcinoma, epithelial-like cell treated with fusion proteins of the invention and comparatively with TRAIL114-281. It can be seen that by administering fusion proteins of the invention of Example 14 and Example 2 PANC-1 tumour growth inhibition was obtained, with TGI respectively 41,5% and 49,8 % relative to the control on 43th day of the experiment. For TRAIL114-281 used as the reference preparation, a slight inhibitory effect on tumour cell growth was obtained relative to the control, with TGI at the level of 32%. Thus, fusion proteins of the invention exert a much stronger effect compared with TRAIL.

The tested fusion proteins did not cause significant side effects manifested by a decrease in body weight of mice (i.e. less than 10% of baseline body weight). This shows a low systemic toxicity of the protein.

Circular dichroizm - determination of secondary structures content in the preparations of fusion proteins of the invention

Quality of the structure the preparations of fusion proteins in terms of their structure was determined by analysis of the secondary structures using circular dichroism (CD). The CD method uses optical activity of the protein structures, manifested in rotating the plane of polarization of light and the appearance of elliptical polarization. CD spectrum of proteins in far ultraviolet (UV) provides precise data on the conformation of the main polypeptide chain.

Samples of the protein prepared in Ex. 1, Ex. 2, Ex. 14, Ex. 24, Ex. 51 and Ex. 42 after were formulation into a buffer consisting of 50 mM Tris-HCl pH 8,0, 100 mM NaCl, 10% glycerol, 0,1 mM $ZnCl_2$, 80 mM saccharose, 5mM DTT were dialysed in the dialysis bags (Sigma-Aldrich) with cut off 12 kDa. Dialysis was performed while stirring against 100 fold excess (v/v) of buffer comparing to the protein preparations, for several hours at 4°C. After dialysis was

completed, each preparation was centrifuged (25 000 rpm., 10 min., 4°C), and the appropriate supernatants were collected. Protein concentration in the samples thus obtained was determined by Bradford method.

Measurement of the circular dichroism for proteins in the concentration range of 0,1-2,7 mg/ml was performed on Jasco J-710 spectropolarimeter, in a quartz cuvette with an optical way 0.2 mm or 1 mm. The measurement was performed under the flow of nitrogen of 7 l/min, which allowed to perform of the measurement in the wavelength range from 195 to 250 nm.

Parameters of the measurement: spectral resolution of - 1 nm; half width of the light beam 1 nm; sensitivity 20 mdeg, the averaging time for one wavelength - 8 s, scan speed 10 nm/min.

The results were presented as the average of three measurements. Circular dichroism spectra for proteins according to the Ex. 1, Ex. 2, Ex. 14, Ex. 24, Ex. 51 and Ex. 42 are presented in Fig. 20.

Obtained spectra were analyzed numerically in the range of 193-250 nm using CDPro pack. Points for which the voltage at the fotomultiplier exceeded 700 V were omitted, due to too low signal to noise ratio in this wavelength range. The data obtained served for calculations of particular secondary structures content in the analyzed proteins with use of CDPro programs package (Table 4).

Table 4. Content of secondary structures in the analyzed proteins

Protein	NRMSD (Exp-Cal)	α-helix (%)	β-sheet (%)	Schift (%)	Disorder (%)
Ex. 24	0.720	4.1%	46.7%	26.4%	22.8%
Ex. 42	0.100	18.4%	28.7%	22.0%	30.8%
Ex. 1	0.105	20.3%	27.4%	22.9%	29.3%
Ex. 2	0.035	14.8%	32.2%	21.3%	31.6%
Ex. 51	0.302	4.5%	38.6%	22.5%	34.4%
Ex. 14	0.220	3.5%	39.0%	21.1%	36.3%
hrTRAIL*		1.94%	50.97%	7.74%	39.35%
hrTRAIL114-281	0.389	4.9%	33.7%	23.1%	38.3%

* value obtained on the basis of crystalline structure 1D4V

Controls (rhTRAIL114-281) reveal a characteristic CD spectrum for the proteins with predominantly type β-sheet structures (sharply outlined ellipticity minimum at the wavelength 220 nm). This confirms the calculation of secondary structure components, which suggests a marginal number of α-

helix elements. The obtained result is also consistent with data from the crystal structure of TRAIL protein, whereby the beta elements constitute more than half of its composition. In the case of hybrid proteins of Ex. 1 and Ex. 42, dichroism spectra are characterized by two minima at wavelengths 208 and 220 nm, which is characteristic for proteins with mixed secondary structure of alpha/beta type. This is probably due to attachment of a domain (e.g. BH3 from Bax) to TRAIL, which forms the alpha-helical structures, so that the mixed nature of secondary structures in the analyzed chimeric proteins can confirm their presence (for Ex. 42 due to poor quality of the spectrum it is less clear).

For preparations of Ex. 2, Ex. 51, Ex. 14 and Ex. 24 as well as for TRAIL protein, a significant content of beta-type structures was found. This is probably due to the fact that attached short peptides initially have the beta structure or are unstructuralized and therefore do not affect significantly their composition. In the case of protein of Ex. 2 a slight increase in the content of alpha structures was also observed. Similarly as with protein of Ex. 1, this may be due to the presence of BH3 domain, which creates similar forms or due to narrow range of wavelengths (high amount of noise in the far-UV excludes readings). The lack of sharply outlined range of 180-200 nm in the analyzed region of the spectrum can cause over-content of α-helix structures.

Claims

1. A fusion protein comprising:

domain (a) which is the functional fragment of soluble hTRAIL protein sequence, wherein said functional fragment is capable of inducing apoptotic signal and begins with an amino acid at a position not lower than hTRAIL95, or a sequence having at least 70% homology thereto; and

domain (b) which is the sequence of a pro-apoptotic effector peptide, which effects its pro-apoptotic action via intrinsic apoptosis pathway, wherein the sequence of domain (b) is attached at the C-terminus and/or the N-terminus of domain (a).

2. The fusion protein according to claim 1 wherein domain (a) is the fragment of soluble hTRAIL protein sequence, which fragment begins with an amino acid from the range hTRAIL95 to hTRAIL121, inclusive, and ends with the amino acid hTRAIL281.

3. The fusion protein according to claim 2, wherein domain (a) is selected from the group consisting of hTRAIL114-281 (SEQ. No. 27), hTRAIL119-281 (SEQ. No. 28), hTRAIL121-281 (SEQ. No. 29), hTRAIL116-281 and hTRAIL120-281.

4. The fusion protein according to claim 1, wherein domain (a) is the sequence hTRAIL95-281.

5. The fusion protein according to claim 1, wherein domain (b) is selected from the group consisting of:

- the fragment of BH3 domain of Bax protein of SEQ. No. 30;
- the fragment of Bid protein of SEQ. No. 31;
- ribonuclease A of SEQ. No. 32;
- cytochrome C of SEQ. No. 33;
- granzyme B of SEQ. No. 34;
- the fragment of Nur77 protein of SEQ. No. 35;
- BH3 domain of Bak protein of SEQ. No. 36;
- BH3 domain of PUMA/BBC3 protein of SEQ. No. 37;
- PUMA/BBC3 protein of SEQ. No. 38;

- the fragment of SMAC/Diablo protein of SEQ. No. 39;
- buforin A of SEQ. No. 40;
- onconase of SEQ. No. 41;
- the fragment of Mdm2 protein of SEQ. No. 42;
- the peptide binding to Mdm2 of SEQ. No. 43;
- a fragment of lunasin of SEQ. No. 44;
- BH3 domain of Bik protein of SEQ. No. 45 ;
- the peptide inhibitor of proteasome of SEQ. No. 46;
- the domain comprising proteasome binding UIM motifs of SEQ. No. 47;
- the azurin derived peptide of SEQ. No. 151;
- the full length azurine peptide of SEQ.No. 152;
- the peptide designed from aPP protein and BH3 domain of Bax protein of SEQ. No. 153;
- the peptide designed from aPP protein and BH3 domain of Bax protein of SEQ. No. 154;
- the Reticulon RTN1-C derived peptide of SEQ. No. 155;
- the full length human Reticulon 3 of SEQ. No. 156;
- the modified constutively active caspase-3 of SEQ. No. 157;
- the SAC domain from Par-4 protein of SEQ. No. 158;
- Noxa protein of SEQ. No. 159;
- MTD/CKP fragment of Noxa protein of SEQ. No. 160;
- the short hybrid peptide Antp-TPR of SEQ. No. 161;
- the peptide inhibitor of the SH2 domain of Stat3 protein of SEQ No. 162;
- the peptide derived from BH3 domain of Bak protein of SEQ. No. 163;
- the peptide derived from BH3 domain of Bad protein of SEQ. No. 164; and
- the peptide ATAP from Bfl1 protein of SEQ. No. 165.

6. The fusion protein according to any of the claims 1 to 5, which between domain (a) and domain (b) contains domain (c) comprising a protease cleavage site recognized by proteases present in the tumour cell environment, selected from a sequence recognized by metalloprotease MMP, a sequence recognized by urokinase uPA, a sequence recognized by furin, and combinations thereof.

7. The fusion protein according to claim 6, wherein the sequence recognized by metalloprotease MMP is SEQ. No. 51, SEQ. No. 171 or SEQ. No. 173, the sequence recognized by urokinase uPA is SEQ. No. 52, and the sequence recognized by furin is SEQ. No. 53 or Seq. No. 172.

8. The fusion protein according to claims 6 or 7, wherein domain (c) is a combination of sequences recognized by metalloprotease MMP and urokinase uPA located next to each other.

9. The fusion protein according to claims 6 or 7, wherein domain (c) is a sequence recognized by furin.

10. The fusion protein according to any of the preceding claims, wherein domain (b) is additionally linked with a transporting domain (d), selected from the group consisting of:

- (d1) sequence directing to endoplasmic reticulum,
- (d2) polyarginine sequence transporting through a cell membrane, comprising 6, 7, 8 or 9 Arg residues,
- (d3) *Pseudomonas aeruginosa* translocation domain selected from SEQ. No. 54 or SEQ. No. 176;
- (d4) a membrane transporting domain,
- (d5) a nuclear localization domain, and
- (d6) a mitochondrial targeting domain,

and combinations thereof.

11. The fusion protein according to claim 10, wherein the sequence (d1) directing to endoplasmic reticulum is KEDL (SEQ. No. 55) or KDEL (SEQ. No. 56).

12. The fusion protein according to claims 10 or 11, wherein the sequence (d1) directing to endoplasmic reticulum is located at the C-terminus of the fusion protein.

13. The fusion protein according to claim 10, wherein the polyarginine sequence (d2) is located at the C-terminus of the fusion protein.

14. The fusion protein according to claim 10, wherein the polyarginine sequence (d2) is located between domains (b) and (c).

15. The fusion protein according to claim 10, wherein *Pseudomonas aeruginosa* translocation domain (d3) is located between domains (a) and (c).

16. The fusion protein according to any one of the preceding claims, which additionally comprises domain (e) of a glycine-serine flexible steric linker between domains (a), (b), (c) and/or (d).

17. The fusion protein according to claim 16, wherein the glycine-serine linker is selected from the group consisting of GGSG (SEQ. No. 57), GGGS (SEQ. No. 58), GGGGS (SEQ. No. 59), GGSGG (SEQ. No. 60), GGGSGG (SEQ. No. 61), GGGSGGG (SEQ. No. 62), GGGSGGGS (SEQ. No. 63), GGGSGGGGS (SEQ. No. 64), ASGG (SEQ. No. 65), GGGSASGG (SEQ. No. 66), GGSHG (SEQ. No. 182), SGCGS (SEQ. No. 169), GGGGSGGGG (SEQ. No. 180), SGGCGGS(SEQ. No. 183) and AACAA (SEQ. No. 184).

18. The fusion protein according to claim 1, having amino acid sequence selected from the group consisting of SEQ. No. 1; SEQ. No. 2; SEQ. No. 3; SEQ. No. 4; SEQ. No. 5; SEQ. No. 6; SEQ. No. 7; SEQ. No. 8 ; SEQ. No. 9; SEQ. No. 10; SEQ. No. 11; SEQ. No. 12; SEQ. No. 13; SEQ. No. 14; SEQ. No. 15; SEQ. No. 16; SEQ. No. 17; SEQ. No. 18; SEQ. No. 19; SEQ. No. 20; SEQ. No. 21; SEQ. No. 22); SEQ. No. 23; SEQ. No. 24; SEQ. No. 25, SEQ. No. 26, SEQ. No. 93, SEQ. No. 94, SEQ. No. 95, SEQ. No. 96, SEQ., No. 97, SEQ. No. 98, SEQ. No. 99, SEQ. No. 100, SEQ. No. 101, SEQ. No. 102, SEQ. No. 103, SEQ. No. 104, SEQ. No. 105, SEQ. No. 106, SEQ. No. 107, SEQ. No. 108, SEQ. No. 109, SEQ. No. 110, SEQ. No. 111, SEQ. No. 112, SEQ. No. 113, SEQ. No. 114, SEQ. No 115, SEQ. No. 116, SEQ. No. 117, SEQ. No. 118, SEQ. No. 119, SEQ. No. 120 and SEQ. No. 121.

19. The fusion protein according to any one of the preceding claims, which is a recombinant protein.

20. The fusion protein according to any one of the preceding claims, which additionally contains as its C-terminal part the sequence hTRAIL95-121, preceded by the sequence of the protease cleavage site allowing its cleavage from the construct.

21. A polynucleotide sequence, coding the fusion protein defined as in any one of claims 1 to 20.

22. The polynucleotide sequence according to claim 21, optimized for genetic expression in *E. coli*.

23. The polynucleotide sequence according to claim 22, selected from the group consisting of SEQ. No. 67; SEQ. No. 68; SEQ. No. 69; SEQ. No. 70; SEQ.

No. 71; SEQ. No. 72; SEQ. No. 73; SEQ. No. 74; SEQ. No. 75; SEQ. No. 76; SEQ. No. 77; SEQ. No. 78; SEQ. No. 79; SEQ. No. 80; SEQ. No. 81; SEQ. No. 82; SEQ. No. 83; SEQ. No. 84; SEQ. No. 85; SEQ. No. 86; SEQ. No. 87; SEQ. No. 88; SEQ. No. 89; SEQ. No. 122; SEQ. No. 123; SEQ. No. 124; SEQ. No. 125; SEQ. No. 126; SEQ. No. 127; SEQ. No. 128; SEQ. No. 129; SEQ. No. 130; SEQ. No. 131; SEQ. No. 132; SEQ. No. 133; SEQ. No. 134; SEQ. No. 135; SEQ. No. 136; SEQ. No. 137; SEQ. No. 138; SEQ. No. 139; SEQ. No. 140; SEQ. No. 141; SEQ. No. 142; SEQ. No. 143; SEQ. No. 144; SEQ. No. 145; SEQ. No. 146; SEQ. No. 147; SEQ. No. 148; SEQ; No. 149; and SEQ. No. 150.

24. A pharmaceutical composition, comprising as an active ingredient the fusion protein as defined in any one of claims 1 to 19 in combination with a pharmaceutically acceptable carrier.

25. A use of the fusion protein as defined in claims 1 to 19 or the pharmaceutical composition as defined in claim 24 for the preparation of a medicament for the treatment of cancer diseases in a mammal, including human.