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 the soluble human TRAIL protein in combination with a sequence of an antiangiogenic 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.

TRAIL protein belonging to the cytokines family (Tumor Necrosis Factor-Related Apoptosis Inducing Ligand), also known as Apo2L (Apo2-ligand), is a potent activator of apoptosis in tumor 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 by 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 these receptors can induce external signaling pathway of suppressor gene p53-independent apoptosis, 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 tumor 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 which acts on a wide range of different types of tumor cells, including hematologic malignancies and solid tumors, while sparing 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 TRAIL and sTRAIL forms 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 with recombinant human soluble TRAIL (rhTRAIL) having amino acid sequence corresponding to amino acids 114-281 of hTRAIL, known under the INN dulanermin, showed its good tolerance and absence of dose limiting toxicity.

Fragment of TRAIL shorter than 114-281 is also able to bind with membrane death receptors and induce apoptosis via these receptors, as recently reported for recombinant circularly permuted mutant of 122-281hTRAIL for example in EP 1 688 498.

Toxic effects of recombinant TRAIL protein on liver cells reported up to now appear to be associated with the presence of modification, i.e. polyhistidine tags, while untagged TRAIL showed no systemic toxicity.

However, in the course of further research and development it appeared that many cancer cells showed primary or acquired resistance to TRAIL (see for example WO2007/022214). Although the mechanism of resistance to TRAIL has not been fully understood, it is believed that it may manifest itself at different levels of TRAIL-induced apoptosis pathway, ranging from the level of cell surface receptors to the executive caspases within the signaling 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 tumors to TRAIL, various combination therapies with radio- and chemotherapeutic agents were designed, 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.

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

One of the targets in cancer therapy is also the inhibition of tumor angiogenesis. Angiogenesis (neouvascularisation) is a pathological, time-unlimited process of developing new blood vessels that supply tumors with oxygen and nutrients. Angiogenesis is indispensable for the growth and expansion of the tumor and promoting its metastasis.

Beneficial effect of inhibition of tumor angiogenesis in cancer therapy is known. Attempts were made to the clinical use of substances that inhibit or regulate the process of angiogenesis, both as a cancer therapy and a complementary cancer therapy.

Inhibitors of angiogenesis are known, both endogenous ones naturally present in the human body and numerous exogenous antiangiogenic substances. Among them there are known proteinaceous inhibitors of angiogenesis, including proteolytic fragments of endogenous proteins. As examples, the protein inhibitors of angiogenesis such as angiostatin (a fragment of plasminogen), endostatin (C-terminal fragment of collagen XVIII), calreticulin, vasostatin - a calreticulin fragment, a fragment of prolactin, a fragment of metalloproteinase 2, or tumstatin - a fragment of collagen IV, can be mentioned (Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest*.

2007;117(9):2362–2368, Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov*. 2007;6:273–286).

For example, tumstatin is a peptide of the size of 28 kDa - a fragment of collagen type IV, capable of binding to integrin αvß3 and preventing angiogenesis by inhibition of endothelial cells proliferation. Moreover, tumstatin independently inhibits activation of Focal Adhesion Kinase (FAK) and phosphatidylinositol 3-kinase PI3 and protein kinase PKB/Akt.

Antiangiogenic activity may be also exerted by inhibition of pro-angiogenic proteins, such as Vascular Endothelial Growth Factor (VEGF), which acts through receptors located on vascular endothelium and which is the main stimulator of neoangiogenesis.

In clinical treatment, including cancer therapy, have already been used as antiangiogenic factors certain substances directed against VEGF, such as monoclonal antibodies bevacizumab and ranibizumab. Other proangiogenic factors stimulating the proliferation and migration of endothelial cells independently of receptors located on the endothelium are also known, which include for example, cytokines such as Platelet-Derived Growth Factors PDGF and epidermal growth factor EGF, TNF, and angiopoietin.

In the process of angiogenesis there is also involved the enzyme aminopeptidase N (APN/CD13), which is a transmembrane metalloprotease. It is known that inhibition of this enzyme may lead to inhibition of neoplastic processes. A number natural and synthetic inhibitors of aminopeptidase N are known. (Bavouis B., Dauzonne D., Aminopeptidase-N/CD13 (EC 3.4.11.2) inhibitors: chemistry, biological evaluations, and therapeutic prospects. Medical Research Review, 2006, 26, (1), 88-130).

Natural inhibitors of APN/CD13 include mainly substances produced by microorganisms. As an representative, among others bestatin, curcumin, and apigenin may be mentioned. It was also found that a short peptide containing CNGRC motif is able to efficiently bind to CD13 (Arap et al., Science, 279:377-380, 1998).

Many of the antiangiogenic substances are currently at different stages of investigations, including clinical trials. However, known therapies aimed at inhibiting angiogenesis have many well-known disadvantages. For example, the

benefits of therapeutic monoclonal antibody bevacizumab in the treatment of breast cancer have been recently questioned. Many antiangiogenic drugs show, for example, a very short half-life, low solubility, poor bioavailability and toxic side effects.

Safety of anti-angiogenic drugs is of special importance because of prolonged use and lack of selectivity of therapy. Strong need for an effective therapeutic and the nature of oncological diseases necessitate a simplified registration procedure for such group of drugs, therefore it is impossible to know all the side effects and drawbacks of the drug. Although, contrary to the chemiotherapeutics, which are directed to all fast proliferating cells, antiangiogenic drugs are directed at different stages of the formation of blood vessels, which results in reduction of the toxicity of therapy. However, there is still a need of anticancer therapy which is aimed at inhibiting angiogenesis while ensuring selectivity against tumor cells. There is therefore a need for new antiangiogenic anticancer therapies with improved toxicological characteristics.

Constructed fusion protein containing sequences of an angiogenesis inhibitor vasostatin and TRAIL114-281 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 of an angiogenesis inhibitor calreticulin and TRAIL114-281 was described as exhibiting apoptosis-inducing effect in tumor cells in CN1609124A.

CN 1256347C discloses fusion protein composed of kininogen D5 60-148 and TRAIl 114-281.

Constructed fusion protein containing sequences of an angiogenesis inhibitor kininostatin, vasostatin and canstatin attached to N- or C-terminus of TRAIL114-281 linked with linker encoding GGGSGGSG are mentioned in Feng Feng-Yi “Phase and Clinical Trial of Rh-Apo2L and Apo2L-Related Experimental Study”, Ph.D. degree thesis, Chinese Peking Union Medical, 2006-10-01; http://www.lw23.com/lunwen_957708432.

Constructed fusion protein containing sequences Tumstatin 183-230 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.

The present invention provides a solution of this problem by providing novel fusion proteins that comprise a domain derived from TRAIL and a short effector peptide domain having the antiangiogenic activity and not including TRAIL fragments, wherein the effector peptide potentiates or complements the action of TRAIL.

Proteins according to the invention are directed selectively to cancer cells, where the individual elements of the protein exert their effects, in particular the effector peptides inhibit tumor angiogenesis. Delivery of the proteins of the invention into the tumour environment allows to minimize the toxicity against healthy cells in the body as well as the side effects and to reduce the frequency of administration. In addition, targeted therapy with the use of proteins according to the invention allows to avoid the problem of low efficiency of previously known nonspecific antiangiogenic therapies caused by low permeability of blood vessels.

Moreover, it turned out that in many cases the fusion proteins of the invention are more potent than soluble TRAIL and its variants including a fragment of the sequence. Until now, known effector peptides used in the fusion protein of the invention were not used in medicine as such because of unfavorable kinetics, rapid degradation by nonspecific proteases or accumulation in the body caused by lack of proper sequence of activation of pathways which are necessary to enable the proper action of the efector peptide at target site. Incorporation of the effector peptide into the fusion protein allows their selective delivery to the site where their action is desirable. Furthermore, the attachment of efector peptide increases the mass of protein, resulting in prolonged half-life and increased retention of protein in the tumour and its enhanced efficiency.

Additionally, in many cases, novel fusion proteins also overcome resistance to TRAIL.

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 , Ex. 5 and Ex. 6..

Fig. 2 presents a schematic structure of fusion proteins of the invention according to Ex. 7, Ex. 8 , Ex. 9, Ex. 10 and Ex. 11 .

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

Fig. 4 shows circular dichroism spectra for rhTRAIL95-281 and fusion proteins of Ex. 1, Ex. 4Ex. 5, Ex. 9 and Ex. 14 expressed in specific ellipticity.

Fig. 5 presents tumor volume changes (% of initial stage) in Crl:CD1-Foxn1nu mice burdened with colon cancer HCT116 treated with fusion proteins of the invention of Ex. 1, Ex. 4, Ex. 5 and Ex. 9 compared to rhTRAIL114-281

Fig. 6 presents the tumor growth inhibition values (%TGI) in Crl:CD1-*Foxn1*^{nu} 1 mice burdened with colon cancer HCT116 treated with fusion proteins of the invention of z Ex. 1, Ex. 4 , Ex. 5 and Ex. 9 compared to rhTRAIL114-281.

Fig. 7 presents tumor volume changes (% of initial stage) in Crl:CD1-Foxn1nu mice burdened with lung cancer A549 treated with fusion proteins of the invention of Ex. 1 compared to rhTRAIL114-281

Fig. 8 presents the tumor growth inhibition values (%TGI) in Crl:CD1-*Foxn1*^{nu} 1 mice burdened with lung cancer A549 treated with fusion proteins of the invention of Ex. 1 compared to rhTRAIL114-281.

Fig. 9 presents a schematic structure of fusion proteins of the invention according to Ex. 16, Ex. 17 , Ex. 18 , and Ex. 19..

Fig. 10 presents tumor volume changes (% of initial stage) in Crl:CD1-Foxn1nu mice burdened with colon cancer HCT116 treated with fusion proteins of the invention from Ex. 5. , Ex. 4. Ex. 9 , and Ex. 1 compared to rhTRAIL114-281.

Fig. 11 presents the tumor growth inhibition values (%TGI) in Crl:CD1-*Foxn1*^{nu} 1 mice burdened with colon cancer HCT116 treated with fusion proteins of the invention from Ex. 5. , Ex. 4. Ex. 9 , and Ex. 1 compared to rhTRAIL114-281.

Fig. 12 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with colon cancer HCT116 treated with fusion proteins of the invention from Ex. 6 and Ex.11 compared to rhTRAIL114-281.

Fig. 13 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with colon cancer HCT116 treated with fusion proteins of the invention from Ex. 6 and Ex.11 compared to rhTRAIL114-281.

Fig. 14 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with colon cancer Colo205 treated with fusion proteins of the invention from Ex. 6 and Ex.19 compared to rhTRAIL114-281.

Fig. 15 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with colon cancer Colo205 treated with fusion proteins of the invention from Ex. 6 and Ex.19 compared to rhTRAIL114-281.

Fig. 16 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with colon cancer SW620 treated with fusion proteins of the invention from Ex. 6 and Ex.11 compared to rhTRAIL114-281.

Fig. 17 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with colon cancer SW620 treated with fusion proteins of the invention from Ex. 6 and Ex.11 compared to rhTRAIL114-281.

Fig. 18 presents tumor volume changes (% of initial stage) in Cby.Cg-foxn1(nu)/J mice burdened with lung cancer A549 treated with fusion protein of the invention from Ex.1 compared to rhTRAIL114-281.

Fig. 19 presents the tumor growth inhibition values (%TGI) in Cby.Cg-foxn1(nu)/J mice burdened with lung cancer A549 treated with fusion protein of the invention from Ex.1 compared to rhTRAIL114-281.

Fig. 20 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer NCI-H460 treated with fusion protein of the invention from Ex. 6 compared to rhTRAIL114-281.

Fig. 21 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer NCI-H460 treated with fusion protein of the invention from Ex. 6 compared to rhTRAIL114-281.

Fig. 22 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer A549 treated with fusion protein of the invention from Ex.5, Ex. 6, Ex. 11 compared to rhTRAIL114-281.

Fig. 23 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer A549 treated with fusion protein of the invention from Ex.5, Ex. 6 , Ex. 11 compared to rhTRAIL114-281.

Fig. 24 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer NCI-H460-Luc2 treated with fusion protein of the invention from Ex.5 compared to rhTRAIL114-281.

Fig. 25 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer NCI-H460-Luc2 treated with fusion protein of the invention from Ex.5 compared to rhTRAIL114-281.

Fig. 26 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer A549 treated with fusion proteins of the invention from Ex.5 and Ex.1 compared to rhTRAIL114-281.

Fig. 27 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with lung cancer A549 treated with fusion proteins of the invention from Ex.5 and Ex.1 compared to rhTRAIL114-281.

Fig. 28 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with liver cancer PLC/PRF/5 treated with fusion proteins of the invention from Ex. 6 and Ex. 11 compared to rhTRAIL114-281.

Fig. 29 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with liver cancer PLC/PRF/5 treated with fusion proteins of the invention from Ex. 6 and Ex. 11 compared to rhTRAIL114-281.

Fig. 30 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with HepG2 liver cancer treated with fusion proteins of the invention from Ex. 6 and Ex.19 compared to rhTRAIL114-281

Fig. 31 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with HepG2 liver cancer treated with fusion proteins of the invention from Ex. 6 and Ex.19 compared to rhTRAIL114-281.

Fig. 32 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with PANC-1 pancreas cancer treated with fusion protein of the invention from Ex. 11 compared to rhTRAIL114-281

Fig. 33 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with PANC-1 pancreas cancer treated with fusion protein of the invention from Ex. 11 compared to rhTRAIL114-281.

Fig. 34 presents tumor volume changes (% of initial stage) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with multidrug-resistant human uterine sarcoma MES-SA/Dx5 treated with fusion proteins of the invention from Ex. 6 and Ex. 19 compared to rhTRAIL114-281.

Fig. 35 presents the tumor growth inhibition values (%TGI) in Crl:SHO-Prkdc^{scid}Hr^{hr} mice burdened with multidrug-resistant human uterine sarcoma MES-SA/Dx5 treated with fusion proteins of the invention from Ex. 6 and Ex. 19 compared to rhTRAIL114-281.

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 amino acid at a position not lower than hTRAIL95 or a homolog of said functional fragment having at least 70% sequence identity, and
- domain (b) which is a sequence of a antiangiogenic effector peptide,

wherein the sequence of the domain (b) is attached at the C-terminus and/or N-terminus of domain (a);

with the proviso that fusion proteins are excluded wherein effector peptide is selected from the group consisting of calreticulin, tumstatin 183-230, kininogen D5, vasostatin, kininostatin and canstatin.

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 in mammalian cells upon binding to its receptors on the surface of the cells.

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

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 or fragment of hTRAIL protein.

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

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 of its linear presentation.

The fusion protein of the invention incorporates at least one domain (b) of the effector peptide, attached at the C-terminus or N-terminus of domain (a).

In a particular embodiment, the domain (a) is a fragment of hTRAIL sequence, beginning with an amino acid from the range of hTRAIL95 to hTRAIL121, inclusive, and ending with the amino acid hTRAIL 281.

In particular, domain (a) may be selected from the group consisting of sequences corresponding to hTRAIL95-281, hTRAIL119-281, hTRAIL120-281 and hTRAIL121-281. It will be evident to those skilled in the art that hTRAIL95-281, hTRAIL119-281, hTRAIL120-281 and hTRAIL121-281 represent a fragment of human TRAIL protein starting with amino acid marked with the number 95, 119, 120 and 121, respectively, in the known sequence of hTRAIL (SEQ. No. 16) published in GenBank under Accession No P50591.

In another particular embodiment, the domain (a) is a homolog of functional fragment of soluble hTRAIL protein sequence beginning at amino acid position

not lower than hTRAIL95 and ending at amino acid hTRAIL281, the sequence of which is at least in 70%, preferably in 85%, identical to original sequence.

In specific variants of this embodiment the domain (a) is a homolog of a fragment selected from the group consisting of sequences corresponding to hTRAIL95-281, hTRAIL114-281, hTRAIL116-281, hTRAIL120-281, hTRAIL121-281 and hTRAIL122-281.

It should be understood that a homolog of a hTRAIL fragment is a variation/modification of the amino acid sequence of this fragment, wherein at least one amino acid is changed, including 1 amino acid, 2 amino acids, 3 amino acids, 4 amino acids, 5 amino acids, 6 amino acids, and not more than 15% of amino acids, and wherein a fragment of the modified sequence has preserved functionality of the hTRAIL sequence, i.e. the ability of binding to cell surface death receptors and inducing apoptosis in mammalian cells. Modification of the amino acid sequence may include, for example, substitution, deletion and/or addition of amino acids.

Preferably, the homolog of hTRAIL fragment having modified sequence shows a modified affinity to the death receptors DR4 (TRAIL-R1) or DR5 (TRAIL-R2) in comparison with the native fragment of hTRAIL.

The term "modified affinity" refers to an increased affinity and/or affinity with altered receptor selectivity.

Preferably, the homolog of the fragment of hTRAIL having modified sequence shows increased affinity to the death receptors DR4 and DR5 compared to native fragment of hTRAIL.

Particularly preferably, the homolog of fragment of hTRAIL having modified sequence shows increased affinity to the death receptor DR5 in comparison with the death receptor DR4, i.e. an increased selectivity DR5/DR4.

Also preferably, the homolog of fragment of hTRAIL having modified sequence shows an increased selectivity towards the death receptors DR4 and/or DR5 in relation to the affinity towards the receptors DR1 (TRAIL-R3) and/or DR2 (TRAIL-R4).

Modifications of hTRAIL resulting in increased affinity and/or selectivity

towards the death receptors DR4 and DR5 are known to those skilled in the art, for example from the publication Tur V, van der Sloot AM, Reis CR, Szegezdi E, Cool RH, Samali A, Serrano L, Quax WJ. DR4-selective tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) variants obtained by structure-based design. J. Biol. Chem. 2008 Jul 18;283(29):20560-8, which describes the D218H mutation having increased selectivity towards DR4, or Gasparian ME, Chernyak BV, Dolgikh DA, Yagolovich AV, Popova EN, Sycheva AM, Moshkovskii SA, Kirpichnikov MP. Generation of new TRAIL mutants DR5-A and DR5-B with improved selectivity to death receptor 5, Apoptosis. 2009 Jun;14(6):778-87, which describes the D269H mutation having a reduced affinity towards DR4. hTRAIL mutants resulting in increased affinity towards one receptor selected from the DR4 and DR5 comparing with DR1 and DR2 receptors and increased affinity towards the receptor DR5 comparing with DR4 are also described in WO2009077857 and WO2009066174.

Suitable mutations are one or more mutations in the positions of native hTRAL selected from the group consisting of 131, 149, 159, 193, 199, 201, 204, 204, 212, 215, 218 and 251, in particular, mutations involving the substitution of an amino acid with a basic amino acid such as lysine, histidine or arginine, or amino acid such as glutamic acid or aspargic acid. Particularly one or more mutations selected from the group consisting of G131R, G131K, R149I, R149M, R149N, R149K, S159R, Q193H, Q193K, N199H, N199R, K201H, K201R, K204E, K204D, K204L, K204Y, K212R, S215E, S215H, S215K, S215D, D218Y, D218H, K251D, K251E and K251Q, as described in WO2009066174, may be specified.

Suitable mutations are also one or more mutations in the positions of native hTRAL selected from the group consisting of 195, 269 and 214, particularly mutations involving the substitution of an amino acid with a basic amino acid such as lysine, histidine or arginine. Particularly one or more mutations selected from the group consisting of D269H, E195R, and T214R, as described in WO2009077857, may be specified.

In a particular embodiment, the domain (a) which is a homolog of the fragment of hTRAIL is selected from D218H mutant of the native TRAIL sequence, as described in WO2009066174, or the Y189N-R191K-Q193R-H264R-I266R-D269H

mutant of the native TRAIL sequence, as described in Gasparian ME, Chernyak BV, Dolgikh DA, Yagolovich AV, Popova EN, Sycheva AM, Moshkovskii SA, Kirpichnikov MP. Generation of new TRAIL mutants DR5-A and DR5-B with improved selectivity to death receptor 5, Apoptosis. 2009 Jun;14(6):778-87.

Domain (b) may be in particular selected from the following group:

- inhibitors of receptors for growth factors selected from receptors for VEGF, PDGF and EGF;

- tumstatin or fragments thereof other than fragment 183-230, and

- inhibitors of aminopeptidase N (CD13).

Within the group of inhibitors of receptors for growth factors the effector peptide of domain (b) may be a fragment of human vascular endothelial growth factor VEGF which binds the VEGF receptor competitively to the natural ligand while being itself devoid of angiogenic activity. As a consequence, angiogenic activity of VEGF is blocked, there is no stimulation of new blood vessels formation and tumor growth is inhibited. In particular, the effector peptide of the above group is the peptide that inhibits the VEGF signal pathway and specifically the 7-amino acid fragment of human VEGF presented by SEQ. No. 17 in the attached Sequence Listing.

It is believed that the peptide comprising sequence of VEGF heptapeptide incorporated into the fusion protein of the invention will effectively eliminate cancer cells by inhibition of angiogenesis process.

Also within the group of inhibitors of receptors for growth factors, the effector peptide of domain (b) may be a fragment of Platelet-Derived Growth Factor PDGF, which binds the PDGF receptor competitively to the natural ligand while being itself devoid of angiogenic activity. As a consequence, angiogenic activity of PDGF is blocked, there is no stimulation of new blood vessels formation and tumor growth is inhibited.

In particular, such an effector peptide is a 19-amino acid peptide – a fragment of PDGF ligand, shown by a sequence of SEQ. No.22 in the attached Sequence Listing.

It is believed that the peptide comprising sequence of Platelet-Derived Growth Factor PDGF protein fragment incorporated into the fusion protein of the

invention will effectively eliminate cancer cells by inhibition of angiogenesis process.

Also within the group of inhibitors of receptors for growth factors, the antiangiogenic effector peptide of domain (b) may be a peptide fragment of Epidermal Growth Factor EGF, which binds the EGF receptor competitively to the natural ligand while being itself devoid of angiogenic activity. As a consequence, angiogenic activity of EGF is blocked, there is no stimulation of new blood vessels formation and tumor growth is inhibited. Such blocking peptides Gly Leu Arg Ser Leu Lys Glu and Gly Leu Arg Ser Leu Arg Glu capable to bind to EGF receptor without activation of intracellular kinase and to block EGR activity are known for example from EP0641358. In particular, such an effector peptide– a fragment of EGF ligand, is shown by a sequence of SEQ. No.23 in the attached Sequence Listing.

It is believed that the peptide comprising sequence of Epidermal Growth Factor EGF incorporated into the fusion protein of the invention will effectively eliminate cancer cells by inhibition of angiogenesis process.

Within the group of tumstatin and its fragments the effector peptide of domain (b) may be a 25-amino acid fragment of tumstatin protein (fragment I), shown by the sequence of SEQ. No.18 in the attached Sequence Listing. The effector peptide of the above presented group is also another 18-amino acids fragment of tumstatin protein (fragment II), shown by a sequence of SEQ. No.19 in the attached Sequence Listing. The antiangiogenic effector peptide of domain (b) may be also a combination of tumstatin peptide fragments, in particular fragment and fragment II located next to each other in any order. In one embodiment, the domain (b) is a combination of fragment I/fragment II (SEQ. No 18/SEQ. No. 19) or a combination of fragment II/fragment I (SEQ. No 19/SEQ. No. 18).

It is believed that the peptide comprising sequence of tumstatin protein fragment I and/or II incorporated into the fusion protein of the invention will effectively eliminate cancer cells by inhibition of angiogenesis process.

The group of inhibitors of the aminopeptidase N/CD13, which bind with enzyme aminopeptidase N/CD13 to inhibit its activity will include short peptides containing motifs NGR or RGD.

Peptides including motives NGR that bind efficiently to aminopeptidase N are described for example by Arap et al., Science, 279:377-380, 1998. On the extracellular domain of aminopeptidase N a fragment exhibiting affinity to RGD motif is also present. Both motifs (RGD and NGR) bind as antagonists with factors involved in the process of neovascularization. Therefore, it is likely that RGD motif resembling NGR motif will bind with aminopeptidase N and consequently act as its inhibitor (Friedlander et al. *Definition of two angiogenic pathways by distinct av integrins. Science (Washington DC), 270: 1500-1502, 1995;* Pasqualini et al Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res. 2000 Feb 1;60(3):722-7).

Within the group of inhibitors of the aminopeptidase N/CD13, the antiangiogenic effector peptide of domain (b) may be a 5-amino acid peptide binding to CD13 shown by SEQ. No.20 in the attached Sequence Listing. Another effector peptide of this group is also 9-amino acids peptide binding to CD13, shown by SEQ. No.21 in the attached Sequence Listing.

It is believed that the peptide comprising sequence of the protein fragment binding with aminopeptidase N/CD13 incorporated into the fusion protein of the invention will effectively eliminate cancer cells by inhibition of angiogenesis process.

The fusion proteins of the invention may comprise more than one effector peptide domain (b), in particular two or three domains (b). In one embodiment the fusion protein of the present invention contains two similar or different effector domains (b) selected from SEQ. No. 17, SEQ. No. 18, SEQ. No. 19, SEQ. No.20 , SEQ. No.21, SEQ. No.22 and SEQ. No.23, wherein the effector domains (b) are located next to each other. In other embodiment the fusion protein of the present invention contains two similar or different effector domains (b) selected from SEQ. No. 17, SEQ. No. 18, SEQ. No. 19, SEQ. No.20 , SEQ. No.21, SEQ. No.22 and SEQ. No.23, wherein the effector domains (b) are located at the N-terminus and/or C-terminus of domain (a).

In particular embodiment the fusion protein of the present invention comprises three effector domains.

As an example, the fusion protein comprises the peptide derived from VEGF (SEQ. No. 17) located at the N-terminus of domain (a) and at the C-terminus of

domain (a) located next to each other fragment I of tumstatin (SEQ. No. 18) and fragment II of tumstatin (SEQ. No. 19).

In specific embodiments of the fusion protein of the invention, the effector peptide is a peptide having antiangiogenic activity selected from the group consisting of SEQ. No. 17 (heptapeptide derived from VEGF), SEQ. No. 18 (a fragment I (aminoacids 74-98) of tumstatin protein), SEQ. No. 19 (a fragment II (aminoacids 197-214) of tumstatin protein), SEQ. No.20 (a peptide binding to CD13), SEQ. No.21 (a peptide binding to CD13), SEQ. No.22 (a fragment of PDGF) and SEQ. No.23 (a fragment of EGF).

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 or its homolog with preserved functionality, 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 of the fusion protein comprising antiangiogenic peptide, the domain (b) will be able to potentially exert its action intracellularly in 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 -such as steric inhibition of binding site of the natural VEGF, PDGF and EGF ligands, inhibition of angiogenesis and neovascularisation, inhibition of activation of phosphatidylinositol 3-kinase, protein kinase B (PKB/Akt) or indirect stimulation of TRAIl overexpression by kinase Akt and NFk pathway.

In one of the embodiments of the invention, domain (a) and domain (b) are linked by at least one domain (c) comprising the sequence of a cleavage site recognized by proteases present in the cell environment, especially in the tumor cell environment. The linkage of the domain (a) with the domain (b) by at least one domain (c) means that between domains (a) and (b) more than one domain (c) may be present, in particular one or two domains (c).

A protease cleavage site can be selected from:

- a sequence recognized by metalloprotease MMP, in particular (Pro Leu Gly Leu Ala Gly Glu Pro/PLGLAGEP) designated as SEQ. No.24, or (Pro Leu Gly Ile Ala Gly Glu /PLGIAGE) designated as SEQ. No.55, or (Pro Leu Gly Leu Ala Gly GluPro /PLGLAGEP) designated as SEQ. No. 56;

- a sequence recognized by urokinase uPA, in particular Arg Val Val Arg (RVVR in one-letter convention) designated as SEQ. No. 25 or a fragment thereof, which with the last amino acid of the sequence to which is attached, forms SEQ. No.25,

and their combinations.

In one of the embodiments of the invention, the protease cleavage site is a combination of the sequence recognized by 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. 24/SEQ. No. 25 or a combination of uPA/MMP SEQ. No. 25/SEQ. No. 24.

In another embodiment, the domain (c) is a combination of MMP/uPA SEQ. No 55 /SEQ. No. 25 or a combination of uPA/MMP SEQ. No. 25/SEQ. No..55.

In another embodiment, the domain (c) is a combination of MMP/uPA SEQ. No 56 /SEQ. No. 25 or a combination of uPA/MMP SEQ. No. 25/SEQ. No..56.

Proteases metalloprotease MMP and urokinase uPA 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), 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.

Apart from the main functional elements of the fusion protein, the cleavage site domain(s), the fusion proteins of the invention may contain a neutral sequence/sequences of a flexible steric glycine-cysteine-alanine linker (spacer). 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 SEQ. No.26 and SEQ. No.27, which are combinations of glycine, cysteine and alanine residues. In another embodiment the flexible steric linker

may be selected from the group consisting of SEQ. No.28, SEQ. No.29, SEQ. No.30 and SEQ. No. 54, consisting of glycine and serine residues. Additionally, the flexible steric linker may be any fragment of SEQ. No.28, SEQ. No.29 SEQ. No.30 and SEQ. No. 54, acting as a flexible steric linker, for example a fragment Gly Gly Gly /GGG or a fragment Gly Gly/GG.

In one embodiment, the flexible steric linker may be also selected from single amino acid residue such as single glutamic acid residue, cysteine, serine, proline or glycine residue.

In other embodiment, the flexible steric linker may be any combination of linkers consisting of SEQ. No.26, SEQ. No.27 SEQ. No.28, SEQ. No.29, SEQ. No.30, SEQ. No. 54 and single amino acids residues of glutamic acid residue, cysteine, serine, proline or glycine.

Particular embodiments of the fusion protein of the invention are fusion proteins comprising an antiangiogenic peptide selected from the group consisting of the proteins represented by SEQ. No.1, SEQ. No.2, SEQ. No.4, SEQ. No.5, SEQ. No.6 and SEQ. No. 46, SEQ. No. 47 and SEQ. No.48, comprising as an effector peptide a heptapeptide derived from VEGF.

Other specific embodiment of the fusion protein of the invention is fusion protein comprising an antiangiogenic peptide selected from the group consisting of the proteins represented by SEQ. No.7 and SEQ. No.8, comprising as an effector peptide sequences binding to CD13.

Other specific embodiment of the fusion protein of the invention is fusion protein comprising an antiangiogenic peptide selected from the group consisting of the proteins represented by SEQ. No.9, SEQ. No.10, SEQ. No.11 and SEQ. No. 49 comprising as an effector peptide a fragment of PDGF.

Other specific embodiment of the fusion protein of the invention is fusion protein comprising an antiangiogenic peptide selected from the group consisting of the proteins represented by No. SEQ. No.12 and SEQ. No.13, comprising as an effector peptide tumstatin and and II fragments.

Other specific embodiment of the fusion protein of the invention is fusion protein comprising an antiangiogenic peptide selected from the group consisting of the proteins represented by SEQ. No.14 and SEQ. No.15, comprising as an effector peptide a fragment of EGF.

Particular embodiments of the fusion protein of the invention are fusion proteins comprising an antiangiogenic peptide selected from the group consisting of the proteins represented SEQ. No.3, comprising as an effector peptide a heptapeptide derived from VEGF, fragment I of tumstatin peptide and fragment II of tumastin peptide.

A detailed description of the structure of representative fusion proteins mentioned above are shown in Figures 1 to 3 and in Fig. 9, 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.

Cosmids, plasmids or modified viruses can be used as expression vectors for the introduction and replication of DNA sequences in host cells. 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-glucuronidase, 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.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; SEQ. No.40; SEQ. No.41, SEQ. No.42; SEQ. No.43 SEQ. No.44; SEQ. No.45, SEQ. No. 50, SEQ. No.51, SEQ. No.52; SEQ. No.53; 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.46; SEQ. No.47; SEQ. No.48; and SEQ. No.49.

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.31 to SEQ. No.45 and SEQ. No 50 to SEQ. No. 53 indicated above, as well as *E. coli* cell 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 or are described in the literature and manuals for laboratory work, such as Maniatis et al., Molecular Cloning. Cold Spring Harbor, N.Y., 1982).

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 hydroxypropyl celulose 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 anti fungal agents, including, for example, and not limited to, parabens, chlorobutanol, himerosal, 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 the 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 a plasmid

Amino acid sequence of the target fusion protein was used as a template to 3generate 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 can be optionally 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. Some target were expressed without any tag, in particular without histidine tah, and those were subsequently purified on SP Sepharose. 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 µM 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 20000 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, if present, was cleaved with thrombin (1:50). After the cleavage, thrombin was separated from the target fusion protein expressed with Histag by purification using Benzamidine SepharoseTM resin. Purification of target fusion proteins expressed without Histag was performed on SP Sepharose. 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 6000 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 8000 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 KH2PO4, 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, if the protein was expressed with histidine tag, 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).

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

The protein of SEQ. No.1 is a fusion protein having the length of 173 amino acids and the mass of 19,8 kDa, in which at the N-terminus of the sequence TRAIL 121-281 heptapeptide derived from VEGF (SEQ. No.17) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL the flexible glycine steric linker (SEQ. No.28) is incorporated.

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.1 and SEQ. No.31, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 1 of the structure described above was used as a template to generate its coding DNA sequence DNA SEQ. No. 31. A plasmid containing the coding sequence of DNA in two versions, one allowing to express His tag and a site recognized by thrombin and the second without any tag, was generated and overexpression of the fusion proteins was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli BL21 (DE3)* and *Tuner(DE3)pLysS* strains, both from Novagen. The proteins were 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 fusion protein having the length of 199 amino acids and the mass of 22,8 kDa, in which at the N-terminus of the sequence TRAIL 95-281 heptapeptide derived from VEGF (SEQ. No.17) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL the flexible glycine steric linker (SEQ. No.28) is incorporated.

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.32, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 2 was used as a template to generate its coding DNA sequence DNA SEQ. No. 32. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* strain 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 fusion protein having the length of 230 amino acids and the mass of 26,3 kDa, in which at the N-terminus of the sequence TRAIL 121-281 heptapeptide derived from VEGF (SEQ. No.17), and at C- terminus of the sequence TRAIL 121-281 fragments I and II of tumstatin (SEQ. No. 18 and SEQ. No. 19, respectively) are attached as an effector peptide. Between the effector peptide attached at the N-terminus of the sequence TRAIL and the sequence of TRAIL there is incorporated the glycine flexible steric linker (SEQ. No. 28). Between the effector peptide attached at the C-terminus of the sequence TRAIL and the sequence of TRAIL there is incorporated steric linker consisting of 3 glycine residues Gly Gly Gly, and sequences of cleavage sites recognized by metalloprotease MMP (SEQ. No. 24) and urokinase uPA (SEQ. No. 25), due to which the effector peptide will undergo cleavage 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. 3 and SEQ. No. 33, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 3 was used as a template to generate its coding DNA sequence SEQ. No. 33. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* strain 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 187 amino acids and the mass of 21,4 kDa, in which at the N-terminus of the sequence TRAIL 121-281 two sequences of heptapeptide derived from VEGF (SEQ. No.17) are attached as an effector peptide. Between the two sequences of effector peptide there is incorporated sequence of cleavage site recognized by metalloprotease MMP (SEQ. No. 24), due to which the effector peptide will undergo cleavage in the tumour environment. Between the sequence of MMP cleavage site and the sequence of effector protein there is incorporated a single glutamic acid residue E. Between the effector peptide (SEQ. 17) and the sequence of TRAIL there is incorporated flexible steric glycine linker (SEQ. No.28).

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.34, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 4 was used as a template to generate its coding DNA sequence DNA SEQ. No. 34. A plasmid containing the coding sequence of DNA in two versions, one allowing to express His tag and a site recognized by thrombin and the second without any tag, was generated and overexpression of the fusion proteins was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure B, using *E. coli BL21DE3pLysSRIL* strain from Stratagene and *Tuner (DE3)* from Novagen. The proteins were 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 187 amino acids and the mass of 21,8 kDa, in which at the N-terminus of the sequence TRAIL 121-281 two sequences of heptapeptide derived from VEGF (SEQ. No.17) are attached as an effector peptide. Between the two sequences of effector

peptides the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 24), due to which the effector peptide will undergo cleavage in the tumour environment. Between the sequence of MMP cleavage site and the sequence of effector protein there is incorporated single glutamic acid residue E. Additionally at the N-terminus of TRAIL two glycine residues are attached.

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.35, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 5 was used as a template to generate its coding DNA sequence DNA SEQ. No. 35. A plasmid containing the coding sequence of DNA in two versions, one allowing to express His tag and a site recognized by thrombin and the second without any tag, was generated and overexpression of the fusion proteins 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 proteins were 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 222 amino acids and the mass of 25,3 kDa, in which at the N-terminus of the sequence TRAIL 95-281 two sequences of heptapeptide derived from VEGF (SEQ. No.17) are attached as an effector peptide. Between the two sequences of effector peptide the protein contains sequences of cleavage sites recognized by uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No.55) due to which the effector peptide will undergo cleavage in the tumour environment. Between the effector peptide (SEQ. 17) and the sequence of TRAIL there is incorporated the cysteine flexible steric linker (SEQ. No.26).

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.6 and SEQ. No.36, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 6 was used as a template to generate its coding DNA sequence DNA SEQ. No. 36 A plasmid containing the coding sequence of DNA, without a sequence allowing to express His tag and a site recognized by thrombin, 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 7. The fusion protein of SEQ. No.7

The protein of SEQ. No.7 is a fusion protein having the length of 168 amino acids and the mass of 19,4 kDa, in which at the N-terminus of the sequence TRAIL 119-281 a sequence being a ligand of CD13 (SEQ. No.20) is attached as an effector peptide.

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.37, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 7 was used as a template to generate its coding DNA sequence DNA SEQ. No. 37. A plasmid containing the coding sequence of DNA in two versions, one allowing to express His tag and a site recognized by thrombin and the second without any tag, was generated and overexpression of the fusion proteins 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 proteins were 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 201 amino acids and the mass of 23,2 kDa, in which at the N-terminus of the sequence TRAIL 95- a sequence being a ligand of CD13 (SEQ. No.21) is attached as an effector peptide. Between the sequence of TRAIL and the sequence of effector peptide the protein contains a sequence of flexible glycine-serine linker (SEQ. No.30).

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.38, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 8 was used as a template to generate its coding DNA sequence DNA SEQ. No. 38. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 9. The fusion protein of SEQ. No.9

The protein of SEQ. No.9 is a fusion protein having the length of 192 amino acids and the mass of 22,1 kDa, in which at the N-terminus of the sequence TRAIL 119-281 a sequence of PDGF fragment (SEQ. No.22) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 24). due to which the effector peptide will undergo cleavage in the tumour environment.

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.39, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 9 was used as a template to generate its coding DNA sequence DNA SEQ. No. 39. A plasmid containing the coding sequence of DNA in two versions, one allowing to express His tag and a site recognized by thrombin and the second without any tag, was generated and overexpression of the fusion proteins was carried out in accordance with the general procedures described above. Overexpression was performed according to the general procedure A, using *E. coli Rosetta (DE3)* strain from Novagen. The proteins were 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 216 amino acids and the mass of 24,9 kDa, in which at the N-terminus of the sequence TRAIL 95-281 a fragment of PDGF (SEQ. No.22) is attached as an effector peptide. Between the sequence of effector peptide and the TRAIL domain the protein contains sequences of cleavage sites recognized by uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 24), due to which the effector peptide will undergo cleavage in the tumour environment.

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.40, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 10 was used as a template to generate its coding DNA sequence DNA SEQ. No. 40. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* and *Tuner(DE3)pLysS* strains, both 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 226 amino acids and the mass of 25,7 kDa, in which at the N-terminus of the sequence TRAIL95-281 a PDGF fragment (SEQ. No.22) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 24). due to which the effector peptide will undergo cleavage in the tumour environment . Between the TRAIL sequence and the sequence of cleavage site recognized by metalloprotease MMP the protein contains also flexible glycine-cysteine-alanine linker (SEQ. No.27).

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.11 and SEQ. No.41, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 11 was used as a template to generate its coding DNA sequence DNA SEQ. No. 41. A plasmid containing the coding sequence of DNA, without a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* and *Tuner(DE3)pLysS* strains, both 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 217 amino acids and the mass of 25 kDa, in which at the N-terminus of the sequence TRAIL 121-281 fragments I and II of tumstatine (SEQ. No.18 and SEQ. No.19) are attached as an effector peptide. Between the effector peptide and the sequence of TRAIL the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 24), due to which the effector peptide will undergo cleavage in the tumour environment. Between the sequence of TRAIL and the sequence of cleavage site recognized by metalloprotease MMP the protein contains also a flexible linker consisting of 3 glycine residues Gly Gly Gly.

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.42, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 12 was used as a template to generate its coding DNA sequence DNA SEQ. No. 42. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* and *Tuner(DE3)pLysS* strains, both from Novagen. 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 220 amino acids and the mass of 25,1 kDa, in which at the N-terminus of the sequence TRAIL 121-281 fragment II of tumstatin (SEQ. No.19) is attached as an effector peptide, and at the C-terminus of the sequence TRAIL 121-281 fragment I of tumstatin (SEQ. No. 18) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 24), due to which the effector peptide will undergo cleavage in the tumour environment. Between the sequence of cleavage site recognized by metalloprotease MMP and the sequence of TRAIL the protein contains three glycine residues Gly Gly Gly and between the C-terminus of the sequence TRAIL and fragment II of tumstatin a flexible linker consisting of 3 glycine residues Gly Gly Gly.

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.43, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 13 was used as a template to generate its coding DNA sequence DNA SEQ. No. 43. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 B.21 (DE3)* strain from Novagen and *BL21DE3pLysSRIL* strain 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 181 amino acids and the mass of 21 kDa, in which at the N-terminus of the sequence TRAIL 120-281 a fragment of EGF (SEQ. No.23) is attached as an effector peptide. Between the effector peptide and the N-terminus of TRAIL domain the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 56), due to which the effector peptide will undergo cleavage in the tumour environment .

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.44, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 14 was used as a template to generate its coding DNA sequence DNA SEQ. No. 44. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* strain from Novagen and *BL21DE3pLysSRIL* strain from Stratagene. 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 217 amino acids and the mass of 24,4 kDa, in which at the N-terminus of the sequence hTRAIL95-281 a fragment of EGF (SEQ. No.23) is attached as an effector peptide. Between the effector peptide and the N-terminus of TRAIL domain the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 24), due to which the effector peptide will undergo cleavage in the tumour environment. Between the sequence of cleavage site recognized by metalloprotease MMP and the sequence of TRAIL the protein contains single proline residue followed by the flexible glycine-cysteine-alanine linker (SEQ. No.26).

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.45, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 15 was used as a template to generate its coding DNA sequence DNA SEQ. No. 45. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 16. The fusion protein of SEQ. No.46

The fusion protein of SEQ. No.46 is a fusion protein having the length of 211 amino acids and the mass of 24,4 kDa, in which at the N-terminus of the sequence TRAIL 95-281 two heptapeptides derived from VEGF (SEQ. No.17) linked to each other are attached as effector peptides. Between the effector peptides the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 55), due to which the effector peptide will undergo cleavage in the tumour environment.

Structure of the fusion protein is shown schematically in Fig. 9, and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in E. coli are, respectively, SEQ. No.46 and SEQ. No.50, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 46 was used as a template to generate its coding DNA sequence DNA SEQ. No.50. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 17. The fusion protein of SEQ. No.47

The fusion protein of SEQ. No.47 is a fusion protein having the length of 200 amino acids and the mass of 22,7 kDa, in which at the N-terminus of the sequence TRAIL 120-281 two heptapeptides derived from VEGF (SEQ. No.17) linked to each other are attached as effector peptides. Between the effector peptides the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 55), due to which the effector peptide will undergo cleavage in the tumour environment. Between the effector protein and the TRAIL domain the protein contains subsequently flexible linker (SEQ. No. 26) promoting trimer formation and flexible glycine –serine linker (SEQ. no. 54).

Structure of the fusion protein is shown schematically in Fig. 9, and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in E. coli are, respectively, SEQ. No.47 and SEQ. No.51, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 47 was used as a template to generate its coding DNA sequence DNA SEQ. No. 51. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 18. The fusion protein of SEQ. No.48

The fusion protein of SEQ. No. 48 is a fusion protein having the length of 192 amino acids and the mass of 21,9 kDa, in which at the N-terminus of the sequence TRAIL 120-281 two heptapeptides derived from VEGF (SEQ. No.17) linked to each other are attached as effector peptides. Between the effector peptides the protein contains sequence of cleavage site recognized by urokinase uPA (SEQ. No. 25), due to which the effector peptide will undergo cleavage in the tumour environment. Between the effector protein and the TRAIL domain the protein contains subsequently flexible linker (SEQ. No. 26) promoting trimer formation and flexible glycine –serine linker (SEQ. no. 54).

Structure of the fusion protein is shown schematically in Fig. 9, and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in E. coli are, respectively, SEQ. No. 48 and SEQ. No.52, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 48 was used as a template to generate its coding DNA sequence DNA SEQ. No. 52. A plasmid containing the coding sequence of DNA, with a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* strain from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 19. The fusion protein of SEQ. No.49

The protein of SEQ. No. 49 is a fusion protein having the length of 206 amino acids and the mass of 23,3 kDa, in which at the N-terminus of the sequence TRAIL120-281 a PDGF fragment (SEQ. No.22) is attached as an effector peptide. Between the effector peptide and the sequence of TRAIL the protein contains sequences of cleavage sites recognized by urokinase uPA (SEQ. No. 25) and metalloprotease MMP (SEQ. No. 55), due to which the effector peptide will undergo cleavage in the tumour environment. Between the TRAIL sequence and the sequence of cleavage site recognized by metalloprotease MMP the protein contains also located subsequently flexible glycine-cysteine-alanine linker (SEQ. No.26) promoting trimer formation and flexible glycine -serine linker (SEQ. no. 54).

Structure of the fusion protein is shown schematically in Fig.9, and its amino acid sequence and the DNA encoding sequence comprising codons optimized for expression in E. coli are, respectively, SEQ. No. 49 and SEQ. No. 53, as shown in the attached Sequence Listing.

The amino acid sequence SEQ. No. 49 was used as a template to generate its coding DNA sequence DNA SEQ. No.53. A plasmid containing the coding sequence of DNA, without a sequence allowing to express His tag and a site recognized by thrombin, 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 BL21 (DE3)* and *Tuner(DE3)pLysS* strains, both from Novagen. The protein was separated by electrophoresis in accordance with the general procedure described above.

Example 20. Examination of anti-tumor activity of the fusion proteins

Examination of anti-tumor activity of the fusion proteins was carried out *in vitro* in a cytotoxicity assay on tumor cell lines and *in vivo* in mice. For comparison purposes, rhTRAIL114-281 protein and placebo were used.

1. Measurement of circular dichroism -determination of secondary structures content of obtained proteins

Quality of the preparations of fusion proteins in terms of their structure was determined by circular dichroism (CD) for Ex. 1, Ex. 4, Ex. 5, Ex. 9 and Ex. 14.

Circular dichroism is used for determination of secondary structures and conformation of protein. 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.

Dialysis

Samples of the protein to be analysed after 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, pH 7,4 (or alternatively 5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutation, 0,1 mM $ZnCl_2$, 10% glycerol, 80 mM sacharose, pH 8,0 for proteins overexpressed as described above but lacking the His-tag and purified on SP Sepharose - marked in the results Table 5 with asterix *) were dialysed in the dialysis bags (Sigma-Aldrich) with cut off 12 kDa. Dialysis was performed against 100 fold excess (v/v) of buffer comparing to the protein preparations with stirring 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 as an average of triplicates.

Determination of protein concentration using Bradford method

In assays of protein concentration of the reagent prepared by dissolving 17.5 mg of Coomassie G-250 in a mixture of ethanol (4.8% v / v) phosphoric acid (V) (5.95% v / v) and water. To determine the protein concentration 1-10 ml of sample was added to 800 ml of Bradford reagent. A reference sample containing Bradford reagent and an appropriate volume of buffer in which the dissolved protein was determined. The absorbance was read on a spectrophotometer Cary 300 at a wavelength of 595 nm after at least 5 minutes incubation of the samples at room temperature. The protein concentration was calculated from the standard curve prepared for the BSA in the range of 10 concentrations 1-10 µg/ml. The starting protein concentration was estimated after taking into account the dilution during the preparation of the sample measurement.

Circular dichroism measurement

Measurement of 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 optical way 0.2 mm or 1 mm. The measurement was performed under the flow of nitrogen at 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, averaging of 3 measurements.

The results were presented as the average of three measurements. Circular dichroism spectra for rhTRAIL114-281 and proteins of Ex. 1, Ex. 4, Ex. 5, Ex. 9 and Ex. 14 are presented in Fig. 4.

Determination of secondary structure content

Obtained spectra were analyzed numerically in the range of 193-250 nm using CDPro software. Points for which the voltage at the photomultiplier 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 software (Table 1).

Table 1. Content of secondary structures in the analyzed proteins

Protein	NRMSD (Exp-Cal)	α-helix	β- sheet	Schift	Disorder
Ex.4	0.319	3.7%	39.4%	20.7%	36.2%
Ex.1	0.093	7.8%	8.6%	63.1%	20.5%
Ex.5	0.04	41.3%	15.0%	2.5%	41.2%
Ex.9	0.112	2.9%	41.0%	20.7%	35.4%
Ex.14	0.244	0.2%	55.3%	17.1%	27.4%
rhTRAIL*		1.94%	50.97%	7.74%	39.35%
rhTRAIL 114-281	0.389	4.9%	33.7%	23.1%	38.3%

* value obtained on the basis of crystalline structure 1D4V

Controls (rhTRAIL114-281) show CD spectrum characteristic 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 hTRAIL protein and characteristic for the proteins of the invention of Ex.4, Ex.

9 and Ex. 14, wherein beta elements constitute more than 40% of its composition.

In the case of all fused proteins, dichroism spectra are characterized by one minimum at wavelength 220 nm. Small effector proteins molecules attached to TRAIL in the fused proteins constitute the minor part of the protein and do not necessarily create a defined secondary structure, the analyzed proteins should not differ significantly from the initial protein. Significant differences, such as high content of alpha structures in the case of protein according to Ex. 5, or sheets such as observed for proteins from Ex. 1 are possibly due to limited range of CD spectrum subjected to analysis, especially in the region 180 - 200 nm.

2. *In vitro* cell line tests

Cell lines

Table 2. Adherent cells

Cell line	Cancer type	Medium	number of cells per well (thousands)
Colo 205 ATCC #CCL-222	*human colorectal cancer*	RPMI + 10% FBS + penicillin + streptomycin	5
HT-29 ATCC # CCL-2	*human colorectal cancer*	McCoy's + 10% FBS + penicillin + streptomycin	5
DU-145 ATCC # HTB-81	*human prostate cancer*	RPMI + 10% FBS + penicillin + streptomycin	3
PC-3 ATCC # CRL-1435	*human prostate cancer*	RPMI + 10% FBS + penicillin + streptomycin	4
MCF-7 ATCC #HTB-22	*human breast cancer*	MEM + 10% FBS + penicillin + streptomycin	4.5
MDA-MB-231 ATCC # HTB-26	*human breast cancer*	DMEM + 10% FBS + penicillin + streptomycin	4.5
UM-UC-3 ATCC # CLR-1749	*human bladder cancer*	MEM + 10% FBS + penicillin + streptomycin	3.5
SW780 ATCC #CRL-2169	*human bladder cancer*	DMEM + 10% FBS + penicillin + streptomycin	3
SW620 ATCC #CCL-227	*human colorectal cancer*	DMEM + 10% FBS + penicillin + streptomycin	5
BxPC-3 ATCC #CRL-1687	*human pancreatic cancer*	RPMI + 10% FBS + penicillin + streptomycin	4.5
NIH: OVCAR-3 ATCC #HTB-161	*human ovarian cancer*	RPMI + 20% FBS + 0,01mg/ml insulin + penicillin + streptomycin	7
HepG2 ATCC # HB-8065	*human liver hepatoma*	MEM + 10% FBS + penicillin + streptomycin	7
293 ATCC # CLR-1573	*Human embrional kidney cells*	MEM + 10% FBS + penicillin + streptomycin	4
ACHN ATCC #CCL-222	*human kidney cancer*	MEM + 10% FBS + penicillin + streptomycin	4
CAKI 2 ATCC # HTB-47	*human kidney cancer*	McCoy's + 10% FBS + penicillin + streptomycin	3.5

Cell line	Cancer type	Medium	number of cells per well (thousands)
HT144 ATCC # HTB-63	*human melanoma cells*	McCoy's + 10% FBS + penicillin + streptomycin	7
LNCaP ATCC # CRL-1740	*human prostate cancer*	RPMI + 10% FBS + penicillin + streptomycin	4.5
NCI-H69 ATCC# HTB-119	*human small cell lung cancer*	RPMI + 10% FBS + penicillin + streptomycin	22
Jurkat A3 ATCC#CRL-2570	*human leukaemia*	RPMI + 10% FBS + penicillin + streptomycin	10
MES-SA/Dx5 ATCC# CRL-1977	uterine cancer	McCoy's + 10% FBS + penicillin + streptomycin	4
SK-MES-1 ATCC# HTB-58	*human lung cancer*	MEM + 10% FBS + penicillin + streptomycin	4
A549 ATCC# CCL-185	*human lung cancer*	RPMI + 20% FBS + penicillin + streptomycin	2.5
HCT116 ATCC# CCL-247	*human colorectal cancer*	McCoy's + 10% FBS + penicillin + streptomycin	3
MCF10A ATCC# CRL-10317	*mammary epithelial cells*	DMEM-F12 (1:1) + 5% horse serum + 0,5µg/ml hydrocortisone + 10µg/ml insulin + 20ng/ml EGF	4.5
MES-SA ATCC# CRL-1976	*uterine cancer*	McCoy's + 10% FBS + penicillin + streptomycin	3.5
PANC-1 CLS# 300228	*human pancreatic cancer*	DMEM + 10% FBS + penicillin + streptomycin	5

Table 3. Nonadherent cells:

Cell line	Cancer type	Medium	Number of cells per well (thousands)
NCI-H69 ATCC# HTB-119	human small cell lung cancer	RPMI + 10% FBS + penicillin + streptomycin	22
Jurkat A3 ATCC#CRL-2570	*human leukaemia*	RPMI + 10% FBS + penicillin + streptomycin	10
HL60 ATCC# CCL-240	*human leukaemia*	RPMI + 20% FBS + penicillin + streptomycin	10
CCRF-CEM ATCC# CCL-119	*human leukaemia*	RPMI + 20% FBS + penicillin + streptomycin	10

MTT cytotoxicity test

MTT assay is a colorimetric assay used to measure proliferation, viability and cytotoxicity of cells. 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 mitochondrial enzyme succinate-tetrazolium reductase 1. MTT reduction occurs only in living cells. Data analysis consists in determining 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 to control cells. Results were analyzed using GraphPad Prism 5.0 software. 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); Involvement 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, and then to the cells (in 100 µl of medium) further 100 µl of the medium containing various concentrations of tested proteins were added. After incubation of the cells with tested proteins over the period of next 72 hours, which is equivalent to 3-4 times of cell division, the medium with the test protein was added with 20 ml of MTT working solution [5 mg/ml], and incubation was continued for 3 h at 37°C in 5% CO_2. Then the medium with MTT solution was removed, and formazan crystals were dissolved by adding 100 µl of DMSO. After stirring, the absorbance was measured at 570 nm (reference filter 690 nm).

EZ4U cytotoxicity test

EZ4U (Biomedica) test was used for testing cytotoxic activity of the proteins in nonadherent cell lines. The test is a modification of the MTT wherein formazan formed in the reduction of tetrazolium salt is water-soluble. Cell viability study was carried out after continuous 72-hour incubation of the cells with protein (seven concentrations of protein, each in triplicates). On this basis IC_{50} values were determined (as an average of two independent experiments) using the GraphPad Prism 5 software.

The results of *in vitro* cytotoxicity tests are summarized in Table 4 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 treated only with solvent.

In Table 4, proteins that were originally expressed with histidine tag that was subsequently removed are designated as a) at the Ex. No.. Proteins that were originally expressed without histidine tag are designated as b) at the Ex. No.. Each experiment represents the average value of at least two independent experiments performed 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 effect/toxicity of the fusion proteins in 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 fusion proteins of the invention into the cells sensitive to TRAIL were administered, in some cases a clear and strong potentiation of the potency of action was observed, manifesting in reduced IC_{50} values of the fusion protein compared with IC_{50} for the TRAIL alone. Furthermore, cytotoxic activity of the fusion protein of the invention in the cells resistant to classical anti-cancer medicament doxorubicin was obtained, and in some cases was stronger than activity of TRAIL alone.

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.

Determination of cytotoxic activity of selected protein preparations against extended panel of tumour cell lines

Table 5 presents the results of the tests 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.

In Table 5, proteins that were originally expressed with histidine tag that was subsequently removed are designated as a) at the Ex. No.. Proteins that were originally expressed without histidine tag are designated as b) at the Ex. No..

Obtained IC_{50} values confirm high cytotoxic activity of fusion protein and thus their potential utility in the treatment of cancer.

Table 4. Cytotoxic activity of fusion proteins of the invention

Protein	Continuous incubation of preparations with cells over 72h (MTT test, 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
rhTRAIL114-281	>2000		32.2	2.40	173	31.3	12.2	2.33	>2000		>2000	
Ex. 9 [a)]	3.96	1.44	3.250	0.95	3.95	9.95	3.00	2.34	131.10	43.98	1420.5	451.22
Ex. 14 [a)]	2000		1738.1	1.47	632.05	26.94	81.27	13.41	2000		2000	
Ex. 7 [a)]	2000		6.822	2.83	38.66	11.34	5.80	1.93	2000		2000	
Ex. 1 [a)]	7.96	0.72	0.743	0.15	25.23	21.98	0.64	0.12	513.10	38.33	131.90	77.92
Ex. 4 [a)]	4.79	0.78	3.69	1.05	14.27	2.48	0.43	0.15	705.15	40.38	>2000	
Ex. 5 [a)]	1.03	0.08	0.699	0.06	2.48	2.03	0.54	0.34	9.95	0.88	13.01	2.17
Ex. 13 [a)]	83.03	21,.74	34.000	3.54	162.00	95.88	22.08	1.43	979.75	1.91	834.05	38.11

Table 5. Analysis of cytotoxic activity of selected protein preparations against broad panel of tumour cell

Cell line	**COLO 205**		**HT 29**		**SW 620**		**MCF 7**		**MDA-MB-231**		**DU 145**		**LNCaP**		**PC 3**	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	24.90	17,68	10000		10000		10000		10000		10000		2052.00	466.0	10000	
Ex. 14 [a)]	3.19	1,68	10000		10000		8839	1642.60	10000		8928.00	543.06	792.70	96.66	10000	
Cell line	**SW 780**		**UM-UC-3**		**293**		**CAKI 2**		**SK-OV-3**		**OV-CAR-3**		**H69AR**		**NCI-H69**	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	120.00	42.43	2242	1367	10000		10000		10000		93.10	8.34	10000		10000	
Ex. 14 [a)]	93.13	33.76	30.37	3.10	8538	2068	10000		10000		190.80	143.17	10000		10000	
Cell line	NCI-H460		BxPC3		HepG2		HT 144		ACHN		JURKAT A3		HL60		CCRF-CEM	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	5889	111.0	64.71	31.81	10000		1734	218.5	10000		10000		10000		10000	
Ex. 14 [a)]	186.80	76.72	79.60	18.81	6153	808.22	1130	26.16	10000		10000		10000		10000	

Table 5, continuation

Cell line	COLO 205		HT 29		SW 620		MCF 7		MDA-MB-231		DU 145		LNCaP		PC 3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	24.90	17.68	10000		10000		10000		10000		10000		2052,00	466,0	10000	
Ex. 1 [a)]	0.87	0.19	852.60	1.06	3650	128.70	832	329.20	23.83	0.54	64.33	22.31	254.00	4.24	980.60	
	SW 780		UM-UC-3		293		CAKI 2		SK-OV-3		OV-CAR-3		H69AR		NCI-H69	
Cell line	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	120.00	42.43	2242	1367	10000		10000		10000		93.10	8,34	10000		10000	
Ex. 1 [a)]	3.78	0.22	7.03	0.13	84350	3.80	230.50	61.50	2116	379	5.58	2.94	1530	137	1436	
	NCI-H460		BxPC3		HepG2		HT 144		ACHN		JURKAT A3		HL60		CCRF-CEM	
Cell line	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	5889	111.0	64.71	31.81	10000		1734	218.5	10000		10000		10000		10000	
Ex. 1 [a)]	7.71	0.09	2.57	0.43	633	89.73	4.47	1.11	71.19	8.92	5.09	2.40	1339		1357	

Table 5, continuation

Cell line	COLO 205		HT 29		SW 620		MCF 7		MDA-MB-231		DU 145		LNCaP		PC 3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	24.90	17.68	10000		10000		10000		10000		10000		2052	466.0	10000	
Ex. 5 [a)]	12.24	3.65	1600		1600		684.50	17.00	345	11,17	473	63.64	1600		1056	
	SW 780		UM-UC-3		293		CAKI 2		SK-OV-3		OV-CAR-3		H69AR		NCI-H69	
Cell line	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	120.00	42.43	2242	1367	10000		10000		10000		93.10	8.34	10000		10000	
Ex. 5 [a)]	38.46	1.03	134.80	9.55	1600		1303	2.10	1600		79.25	27.93	1600		1600	
	NCI-H460		BxPC3		HepG2		HT 144		ACHN		JURKAT A3		HL60		CCRF-CEM	
Cell line	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	5889	111.0	64.71	31.81	10000		1734	218.5	10000		10000		10000		10000	
Ex. 5 [a)]	118.90	28.14	93.90	1.41	1315	389.62	57.44	1.89	510.00	76.37	30.15	4,00	1600		1600	

Table 5, continuation

Cell line	COLO 205		HT 29		SW 620		MCF 7		MDA-MB-231		DU 145		LNCaP		PC 3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	24.90	17.68	10000		10000		10000		10000		10000		2052	466.0	10000	
Ex. 9 [a)]	0.013	0.01	264.20	46.95	47.86	12,50	1025	190.10	1.276	0.40	15.77	9.81	32.90	27.01	463.90	
Cell line	SW 780		UM-UC-3		293		CAKI 2		SK-OV-3		OV-CAR-3		H69AR		NCI-H69	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	120.00	42.43	2242	1367	10000		10000		10000		93.10	8.34	10000		10000	
Ex. 9 [a)]	1.006		0.136	0.07	181.60	44.50	24.42	0.10	2500		0.456	0.64	818.60	130.67	2500	
Cell line	NCI-H460		BxPC3		HepG2		HT 144		ACHN		JURKAT A3		HL60		CCRF-CEM	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	5889	111.0	64.71	31.81	10000		1734	218.5	10000		10000		10000		10000	
Ex. 9 [a)]	0.004	0.01	0.001		9.78	1.31	0.845	1.20	4.46	1.98	0.615	1.00	2500		2500	

Table 5, continuation

Cell line	COLO 205		HT 29		SW 620		MCF 7		MDA-MB-231		DU 145		LNCaP		PC 3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	24.90	17.68	10000		10000		10000		10000		10000		2052.00	466.0	10000	
Ex. 7 [a)]	3.04	0.32	8500		8500		8500		58.00	2.12	4062	1109	3250	766.50	8500	
Cell line	SW 780		UM-UC-3		293		CAKI 2		SK-OV-3		OV-CAR-3		H69AR		NCI-H69	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	120.00	42.43	2242	1367	10000		10000		10000		93.10	8.34	10000		10000	
Ex. 7 [a)]	7.01	2.58	7.63	0.51	6767	2188	8500		8500		15.14	2.62	8500		8500	
Cell line	NCI-H460		BxPC3		HepG2		HT 144		ACHN		JURKAT A3		HL60		CCRF-CEM	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	5889	111.0	64.71	31.81	10000		1734	218.5	10000		10000		10000		10000	
Ex. 7 [a)]	7.11	1.52	7.94	3.19	8500		92.05	40.52	8500		8500		8500		8500	

Table 5, continuation

Cell line	A549		HCT116		MCF10A		MES-SA/Dx5		SK-MES-1							
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD						
rhTRAIL 95-281	>10000		7557	3454	>10000		29.15	12.66	39.35	8.13						
Ex. 9 [b)]	391.00	52.33	3.44		1169		<0.001		3.58	0.81						

Cell line	A549		HCT116		MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1		NCI-H460			
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD		
rhTRAIL 95-281	>10000		7557	3454	>10000		29.15	12.66	29.15	12.66	39.35	8.13	5889	111		
Ex. 16 [a)]	224.84	268.26	2473	500	99.27	51.24	0.36	0.25	0.007	0.00	5		22.76			

Cell line	A549		HCT116		MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1		HT29		NCI-H460	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	>10000		7557	3454	>10000		29.15	12.66	29.15	12.66	39.35	8.13	>10000		5889	111
Ex. 6 [a)]	422.70		0.014		129.90		0.01		0.0068	0.0043	1.41		69.19	18.79	0.02	

Table 5, continuation

Cell line	PANC1		PLC/PRF/5		Colo 205		HepG2		BxPc3		SW 620					
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD				
rhTRAIL 95-281	>10000		>9000		24.90	17.68	>10000		64.71	31.81	>10000					
Ex. 6 [a)]	2.15	0.79	2.35		0.003		0.062		0.014		398.80	80.89				
Cell line	A549		MCF10A		MES-SA/Dx5		SK-MES-1		PANC1		293		UM-UC-3			
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD		
rhTRAIL 95-281	>10000		>10000		29.15	12.66	39.35	8.13	>10000		>10000		2242	1367		
Ex. 1 [b)]	346.75	102.18	147.80	3.96	4.677	2.23	3.29	1.07	12.38	4.20	84.50	3.82	7.03	0.13		
Cell line	A549		HCT116		MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1		HT29		NCI-H460	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	>10000		7557	3454	>10000		>10000		29.15	12.66	39.35	8.13	>10000		5889	111
Ex. 11 [a)]	106.66	41.49	11.50	3.42	95.44		5.28	3.50	0.445	0.30	4.99		911.50	282.14	9.34	5.27

Table 5, continuation

Cell line	PANC1		PLC/PRF/5													
	mean	SD	mean	SD												
rhTRAIL 95-281	>10000		>9000													
Ex. 11 a)	3.07		<0.001													
Cell line	A549		HCT116		MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1		NCI-H460		Colo 205	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	>10000		7557	3454	>10000		>10000		29.15	12.66	39.35	8.13	5889	111	24.90	17.68
Ex. 19 a)	4.31		<0.001		0.19		<0.001		<0.001		<0.001		<0.001		0.004	
Cell line	HepG2		BxPc3				Cell line		HCT116		MCF10A		MES-SA/Dx5		SK-MES-1	
	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	>10000		64.71	31.81			rhTRAIL 95-281		7557	3454	>10000		29.15	12.66	39.35	8.13
Ex. 19 a)	0.20		0.013				Ex. a)		58.86		306.05	7.00	3.492	0.07	9.13	1.31

Table 5, continuation

Cell line	A549		HCT116		MCF10A		MES-SA		SK-MES-1		Cell line	HCT116		BxPc3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD		mean	SD	mean	SD
rhTRAIL 95-281	>10000		7557	3454	>10000		>10000		39.35	8.13	rhTRAIL 95-281	7557	3454	64.71	31.81
Ex. 13 a)	979.75	1.91	162.00	12.45	834.05	38.11	83.03	21.74	22.08	1.43	Ex. 14 a)	275.10	67.46	79.6	18.81

Cell line	A549		HCT116		MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1					
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD				
rhTRAIL 95-281	>10000		7557	3454	>10000		>10000		29.15	12.66	39.35	8.13				
Ex. 17 a)	233.90		30.33		174.10		7.4		1.589		2.9					

Cell line	A549		HCT116		MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1					
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD				
rhTRAIL 95-281	>10000		7557	3454	>10000		>10000		29.15	12.66	39.35	8.13				
Ex. 18 a)	34.28	15.04	11.67	2.39	19.29	4.33	1.24	0.97	0.264	0.35	0.615					

Table 5, continuation

Cell line	HCT116		MES-SA		SK-MES-1		HT29		NCI-H460		PANC1		PLC/PRF/5		Colo 205	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	7557	3454	>10000		39.35	8.13	>10000		5889	111	>10000		>9000		24.90	17.68
Ex. 5 [b)]	0.036	0.01	0.005	0.007	0.004	0.005	783,50	34,65	0.25	0,25	1.05	0.56	5.54		12.24	3.65

Cell line	HepG2		BxPc3		ACHN		OV-CAR-3				Cell line	DU 145		OV-CAR-3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD		mean	SD	mean	SD
rhTRAIL 95-281	>10000		64.71	31.81	>10000		963.00	144.25			rhTRAIL 95-281	>10000		963	144.25
Ex. 5 [b)]	9.27		0.36	0.44	0.5	0.09	<0.001	0.001			Ex. 7 [a)]	4061	1109	15.14	2.62

Cell line	HCT116		MES-SA/Dx5		SK-MES-1		NCI-H460		Colo 205		BxPc3		SW 780		UM-UC-3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	7557	3454	29.15	12.66	39.35	8.13	5889	111	24.90	17.68	64.71	31.81	120	42.43	2242	1367
Ex. 7 [b)]	7.99	1.20	6.822	2.83	5.80	1.93	7.11	1.52	3.04	0.32	7.93	3.19	7.01	2.58	7.63	0.51

Table 5, continuation

Cell line	MCF10A		MES-SA		MES-SA/Dx5		SK-MES-1		HT29		NCI-H460		PANC1		PLC/PRF/5	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	>10000		>10000		29.15	12,66	39.3	8.1	>10000		5889	111	>10000		>9000	
Ex. 9 [b)]	10.30	4.15	<0.001		0.008		0.02		264.2	46.9	0.87	0.01	0.025	0.035	21.87	3.58

Cell line	ACHN		SW 780		UM-UC-3		Cell line	PANC1		PLC/PRF/5		NCI-H460		PANC1	
	mean	SD	mean	SD	mean	SD		mean	SD	mean	SD	mean	SD	mean	SD
rhTRAIL 95-281	>10000		120	42.43	2242	1367	rhTRAIL 95-281	>10000		>9000		5889	111	>10000	
Ex. 9 [b)]	4.46	1.98	<0.001		0.14	0.07	Ex. 15 [a)]	128.00		3722		15.20		128	

Cell line	HCT116		MES-SA		MES-SA/Dx5		SK-MES-1		PC 3				Cell line	UM-UC-3	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD		mean	SD
rhTRAIL 95-281	7557	3454	>10000		29.15	12.66	39.3	8.13	>10000				rhTRAIL 95-281	2242	1367
Ex. 4 [b)]	14.27	2,48	4.79	0.78	3.69	1.05	0.43	0.15	1056	180.9			Ex. 14 [a)]	30.37	3.10

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 HCT116, Colo205 and SW620 cells, human non-small cell lung cancer A549 and NCI-H460-Luc2 cells, human hepatoma PLC/PRF/5 (CLS) cells, human pancreatic carcinoma PANC-1 cells, human liver carcinoma HepG2 cells, human large-cell lung carcinoma NCI-H460 cells, and human uterine carcinoma MES-SA/Dx5 multidrug resistant cells.

Cells

The HCT116 and A549 (ATCC CCL-185) cells 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 $25x10^6$ cells/ml.

The PLC/PRF/5 (CLS), SW620 and PANC-1 cells were maintained in DMEM (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 $25x10^6$ cells/ml.

The HepG2 cells were maintained in MEM (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 $25x10^6$ cells/ml.

The NCI-H460-Luc2, NCI-H460 and Colo205 were maintained in RPMI1640 (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 $25x10^6$ cells/ml.

The MES-SA/Dx5 cells were maintained in McCoy's (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 $25x10^6$ cells/ml.

Mice

Examination of antitumor activity of proteins of the invention was conducted on 4-5 week-old or 7-9 week-old CD- nude (Crl:CD1-*Foxn1*^{nu} 1) or on 4-5 week old Crl:SHO-Prkdc^{scid}Hr^{hr} mice obtained from Charles River Germany or 4-5 week-old Cby.Cg-foxn1(nu)/J mice obtained from Centrum Medycyny Doświadczalnej in Białystok. 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 experiments

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

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

wherein WT refers to the average tumor volume in the treatment group, WC refers to the average tumor 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 GraphPad Prism 5.0 software.

Human colon cancer model

mice Crl:CD1-*Foxn1*^{nu} 1

On day 0 mice Crl:CD1-*Foxn1*^{nu} 1 were grafted subcutaneously (sc) in the right side with 5x10^6 of HCT116 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 60-90 mm^3 (day 14), mice were randomized to obtain the average size of tumors in the group of ~ 70 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention of Ex. 1 (10 mg/kg), Ex. 4 (10 mg/kg),, Ex. 5 (10 mg/kg), and Ex. 9 (10 mg/kg), and rhTRAIL114-281 (10 mg/kg) as a comparison. The preparations were administered intravenously (*i.v.*) daily for ten days. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:CD1-*Foxn1*^{nu} burdened with HCT116 colon cancer treated with fusion proteins of the invention of Ex. 1 , Ex. 4, Ex. 5 and Ex. 9 and comparatively with rhTRAIL114-281 are shown in Fig. 5 as a diagram of changes of the tumor volume and in Fig. 6 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 5 and 6 show that administration of the fusion proteins of the invention of Ex. 1, Ex. 4, Ex. 5 and Ex. 9 caused tumor HCT116 growth inhibition, with TGI respectively 67.8; 69.8; 84.4 and 66.2% relative to the control on 27th day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 44%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Mice Crl:CD1-*Foxn1*^{nu}

HT116 model

On day 0 mice Crl:CD1-*Foxn1*^{nu} were grafted subcutaneously (sc) in the right side with 5x10^6 of HCT116 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of

~ 50-78 mm^3 (day 8), mice were randomized to obtain the average size of tumors in the group of ~ 63 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention of Ex. 5. (10 mg/kg), Ex. 4. (10 mg/kg), Ex. 9 (10 mg/kg), Ex. 1 (10 mg/kg) and rhTRAIL114-281 (10 mg/kg) as a comparison against formulation buffer (50 mM Trizma Base, 150 mM NaCl, 80 mM Saccharose, 250 mM L-arginine, 1 mM glutation, Zn^{2+} 0.1 mM, pH 7.3) as a control. The preparations were administered intravenously (*i.v.*) daily for five days, followed by (after 2-days break) another five daily administrations. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:CD1-*Foxn1*^{nu} burdened with HCT116 colon cancer treated with fusion proteins of the invention of Ex. 5. , Ex. 4, Ex. 9 , Ex. 1 and comparatively with rhTRAIL114-281 are shown in Fig. 10 as a diagram of changes of the tumor volume and in Fig. 11 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 10 and 11 show that administration of the fusion proteins of the invention of Ex. 5. , Ex. 4. , Ex. 9 , and Ex. 1 caused tumor HCT116 growth inhibition, with TGI respectively 80%, 79%, 66% and 68% relative to the control on 27th day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 44.3%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Mice Crl:SHO-Prkdc^{scid}Hr^{hr}

HT116 model

On day 0 mice Crl:SHO-Prkdc^{scid}Hr^{hr} were grafted subcutaneously (sc) in the right side with 5x10^6 of HCT116 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 380-430 mm^3 (day 14), mice were randomized to obtain the average size of tumors in the group of ~ 400 mm^3 and assigned to treatment groups. The

treatment groups were administered with the preparations of fusion proteins of the invention of Ex. 6 (30 mg/kg), Ex.11 (45 mg/kg) and rhTRAIL114-281 (20 mg/kg) as a comparison against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0.1 mM $ZnCl_2$, 10% glycerol, 80 mM saccharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ burdened with HCT116 colon cancer treated with fusion proteins of the invention of Ex. 6 (30 mg/kg), Ex.11 (45 mg/kg) and comparatively with rhTRAIL114-281 are shown in Fig. 12 as a diagram of changes of the tumor volume and in Fig. 13 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figs 12 and 13 show that administration of the fusion proteins of the invention of Ex. 6 and Ex.11 caused tumor HCT116 growth inhibition, with TGI respectively 42% and 44.5% relative to the control on 32^{nd} day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 5.6%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$

<u>COLO205 model</u>

On day 0 mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ were grafted subcutaneously (sc) in the right side with $5x10^6$ of Colo205 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 90-130 mm^3 (day 13), mice were randomized to obtain the average size of tumors 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 of Ex. 6 (30 mg/kg), Ex. 19 (30 mg/kg) and rhTRAIL114-281 (30 mg/kg) as a comparison against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0,1 mM $ZnCl_2$, 10% glycerol, 80 mM

saccharose, pH 8,0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ burdened with Colo205 colon cancer treated with fusion proteins of the invention of Ex. 6 (30 mg/kg), Ex. 19 (45 mg/kg) and comparatively with rhTRAIL114-281 are shown in Fig. 14 as a diagram of changes of the tumor volume and in Fig. 15 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 14 and 15 show that administration of the fusion proteins of the invention of Ex. 6 and Ex. 19 caused tumor Colo205 growth inhibition, with TGI respectively 100% and 100% relative to the control on 33^{th} day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 18.8%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$

SW620 model

On day 0 mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ were grafted subcutaneously (sc) in the right side with $5x10^6$ of SW620 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 290-350 mm^3 (day 17), mice were randomized to obtain the average size of tumors in the group of ~ 320 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention of Ex. 6 (30 mg/kg), Ex.11 (40mg/kg) and TRAIL114-281 (30 mg/kg) as a comparison against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0.1 mM $ZnCl_2$, 10% glycerol, 80 mM saccharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor

size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ burdened with SW620 colon cancer treated with fusion proteins of the invention of Ex. 6 Ex.11 and comparatively with rhTRAIL114-281 are shown in Fig. 16 as a diagram of changes of the tumor volume and in Fig. 17 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 16 and 17 show that administration of the fusion proteins of the invention of Ex. 6 and Ex.11 caused tumor SW620 growth inhibition, with TGI respectively 62% and 23 relative to the control on 31st day of the experiment. For rhTRAIL114-281 used as the comparative reference, no inhibitory effect on tumor 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 rhTRAIL114-281 alone.

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.

Human lung cancer model

Mice Crl:CD1-*Foxn1*^{nu} 1

On day 0 mice Crl:CD1-*Foxn1*^{nu} 1 were grafted subcutaneously (sc) in the right side with 5x10^6 of A549 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~80-100 mm^3 (day 14), mice were randomized to obtain the average size of tumors in the group of ~90 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparation of fusion protein of the invention of Ex. 1(10 mg/kg), and rhTRAIL114-281 (10 mg/kg as a comparison. The preparations were administered intravenously (*i.v.*) every second day for twelve days. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:CD1-*Foxn1*^{nu} burdened with A549 lung cancer treated with fusion proteins of the invention of Ex. 1 and comparatively with rhTRAIL114-281 are shown in Fig. 7 as a diagram of changes of the tumor volume and in Fig. 8 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 7 and 8 show that administration of the fusion protein of the invention of Ex. 1 caused tumor A549 growth inhibition, with TGI 44.8% relative to the control on 33th day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 16.5%. Thus, fusion proteins of the invention exert much stronger effect compared to TRAIL alone.

Cby.Cg-foxn1(nu)/J

On day 0 mice Cby.Cg-foxn1(nu)/J were grafted subcutaneously (sc) in the right side with 5x10^6 of A549 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 60-90 mm^3 (day 19), mice were randomized to obtain the average size of tumors in the group of ~ 75 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion protein of the invention of Ex.1 (15 mg/kg) and rhTRAIL114-281 (20 mg/kg) as a comparison against water for injection as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Cby.Cg-foxn1(nu)/J burdened with A549 lung cancer treated with fusion protein of the invention of Ex.1 and comparatively with rhTRAIL114-281 are shown in Fig. 18 as a diagram of changes of the tumor volume and in Fig. 19 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 18 and 19 show that administration of the fusion protein of the invention Ex.1 caused tumor

A549 growth inhibition, with TGI 44.8% relative to the control on 33th day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 16.6%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Mice: Crl:SHO-Prkdc^{scid}Hr^{hr}

A. On day 0 mice Crl:SHO-Prkdc^{scid}Hr^{hr}were grafted subcutaneously (sc) in the right side with 5x10^{6} of NCI-H460 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 150-170 mm^{3} (day 13), mice were randomized to obtain the average size of tumors in the group of ~ 160 mm^{3} and assigned to treatment groups. The treatment groups were administered with the preparations of fusion protein of the invention of Ex. 6 (30 mg/kg) and rhTRAIL114-281 (30 mg/kg) as a comparison against against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0,1 mM $ZnCl_2$, 10% glycerol, 80 mM saccharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^{3}, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-Prkdc^{scid}Hr^{hr} burdened with NCI-H460 lung cancer treated with fusion protein of the invention of Ex. 6 and comparatively with rhTRAIL114-281 are shown in Fig. 20 as a diagram of changes of the tumor volume and in Fig. 21 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 20 and 21 show that administration of the fusion protein of the invention Ex. 6 caused tumor NCI-H460 growth inhibition, with TGI 88.5% relative to the control on 28th day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 17.5%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

B.On day 0 mice Crl:SHO-Prkdc^{scid}Hr^{hr}were grafted subcutaneously (sc) in the right side with 7x10^6 of A549 cells suspended in a mixture of 0.2 ml HBSS:Matrigel in ratio 3:1 by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 140-165 mm^3 (day 19), mice were randomized to obtain the average size of tumors in the group of ~ 150 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention of Ex.5 (60 mg/kg), Ex. 6 (50 mg/kg), Ex. 11(50 mg/kg) and rhTRAIL114-281 (20 mg/kg) as a comparison against against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0.1 mM $ZnCl_2$, 100 mM L-arginine, 80 mM sacharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-Prkdc^{scid}Hr^{hr} burdened with A549 lung cancer treated with fusion proteins of the invention of Ex.5, Ex. 6, , Ex. 11 and comparatively with rhTRAIL114-281 are shown in Fig. 22 as a diagram of changes of the tumor volume and in Fig. 23 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 22 and 23 show that administration of the fusion proteins of the invention Ex.5, Ex. 6, , and Ex. 11 caused tumor A549 growth inhibition, with TGI respectively 39.3%, 39.3% and 28% relative to the control on 38th day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 5.3%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

C. On day 0 mice Crl:SHO-Prkdc^{scid}Hr^{hr} were grafted subcutaneously (sc) in the right side with 7x10^6 of NCI-H460-Luc2 cells suspended in 0.1 ml HBSS by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 100-120 mm^3 (day 19), mice were randomized to obtain the average size of tumors in the group of ~ 110mm^3 and assigned to treatment groups. The

treatment groups were administered with the preparations of fusion protein of the invention of Ex.5 (first administration 40 mg/kg, followed by 30 mg/kg), and rhTRAIL114-281 (20 mg/kg) as a comparison against against formulation buffer (19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 50 mM NaCl, 5 mM glutation, 0.1 mM $ZnCl_2$, 10% glycerol, pH 7.4) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ burdened with NCI-H460-Luc2 lung cancer treated with fusion protein of the invention of Ex.5 and comparatively with rhTRAIL114-281 are shown in Fig. 24 as a diagram of changes of the tumor volume and in Fig. 25 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 24 and 25 show that administration of the fusion protein of the invention of Ex.5 caused tumor NCI-H460-Luc2 growth inhibition, with TGI 97.2% relative to the control on 29^{th} day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 76%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

D. On day 0 mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ were grafted subcutaneously (sc) in the right side with $7x10^6$ of A549 cells suspended in 0.1 ml mixture of HBSS:Matrigel by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of 100-120mm^3 (day 17), mice were randomized to obtain the average size of tumors in the group of ~ 110mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention of Ex.5 (50 mg/kg), Ex.1 (50 mg/kg), and rhTRAIL114-281 (20 mg/kg) as a comparison against against formulation buffer (19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 50 mM NaCl, 5 mM glutation, 0.1 mM $ZnCl_2$, 10% glycerol, pH 7.4) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average

tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ burdened with A549 lung cancer treated with fusion proteins of the invention of Ex.5 , Ex.1 and comparatively with rhTRAIL114-281 are shown in Fig. 26 as a diagram of changes of the tumor volume and in Fig. 27 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 26 and 27 show that administration of the fusion proteins of the invention of Ex.5 and Ex.1 caused tumor A549 growth inhibition, with TGI respectively 52.5% and 41.6% relative to the control on 34^{th} day of the experiment. For rhTRAIL114-281 used as the comparative reference, a slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 21.8%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Liver cancer model

Mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$A. On day 0 mice Crl:SHO-$Prkdc^{scid}Hr^{hr}$ were grafted subcutaneously (sc) in the right side with $5x10^6$ of PLC/PRF/5 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 190-220 mm^3 (day 31), mice were randomized to obtain the average size of tumors in the group of ~ 200 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion protein of the invention of Ex. 6 (40 mg/kg) and Ex.11 (50 mg/kg), and rhTRAIL114-281 (30 mg/kg) as a comparison against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0.1 mM $ZnCl_2$, 10% glycerol, 80 mM saccharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) following the schema: 4 administration every third day and 2 administrations every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-Prkdc^{scid}Hr^{hr} burdened with PLC/PRF/5 liver cancer treated with fusion proteins of the invention of Ex. 6 and Ex.11 and comparatively with rhTRAIL114-281 are shown in Fig. 28 as a diagram of changes of the tumor volume and in Fig. 29 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 28 and 29 show that administration of the fusion proteins of the invention Ex. 6 and Ex.11 caused tumor PLC/PRF/5 growth inhibition, with TGI respectively 70.6% and 63.8% relative to the control on 49th day of the experiment. For rhTRAIL114-281 used as the comparative reference, the inhibitory effect on tumor cell growth was not obtained relative to the control, with TGI at the level of -18%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Mice Crl:SHO-Prkdc^{scid}Hr^{hr}

A. On day 0 mice Crl:SHO-Prkdc^{scid}Hr^{hr} were grafted subcutaneously (sc) in the right side with 5x10^6 of HepG2 cells suspended in 0.2 ml HBSS buffer by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 190-220 mm^3 (day 31), mice were randomized to obtain the average size of tumors in the group of ~ 200 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion protein of the invention of Ex. 6 (30 mg/kg), Ex.19 (30 mg/kg) and rhTRAIL114-281 (30 mg/kg) as a comparison against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0,1 mM $ZnCl_2$, 10% glycerol, 80 mM saccharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-Prkdc^{scid}Hr^{hr} burdened with HepG2 liver cancer treated with fusion proteins of the invention of Ex. 6 , Ex. 19 and comparatively with rhTRAIL114-281 are shown in Fig. 30 as a diagram of changes of the tumor volume and in Fig. 31 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 30 and 31 show that administration of the fusion proteins of the invention Ex. 6 and Ex.19 caused tumor HepG2 growth inhibition, with TGI respectively 82.6% and 43% relative to the control on 33th day of the experiment. For rhTRAIL114-281 used as the comparative reference, the slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 12.6%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Pancreas cancer model

On day 0 mice Crl:SHO-Prkdc^{scid}Hr^{hr} were grafted subcutaneously (sc) in the right side with 7x10^6 of PANC1 cells suspended in 0.1 ml of HBSS:Matrigel 3:1 mixture by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 87-110mm^3 (day 27), mice were randomized to obtain the average size of tumors in the group of ~ 95 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparation of fusion protein of the invention of Ex. 11 (50 mg/kg) and rhTRAIL114-281 (20 mg/kg) as a comparison against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutation, 0.1 mM $ZnCl_2$, 100 mM L-arginine, 80 mM saccharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-Prkdc^{scid}Hr^{hr} burdened with PANC1 pancreas cancer treated with fusion protein of the invention of Ex. 11and comparatively with rhTRAIL114-281 are shown in Fig. 32 as a diagram of changes of the tumor volume and in Fig. 33 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 32 and 33 show that administration of the fusion protein of the invention Ex. 11 caused tumor PANC1 growth inhibition, with TGI 43% relative to the control on 40th day of the experiment. For rhTRAIL114-281 used as the comparative reference, the slight inhibitory effect on tumor cell growth was obtained relative to the control, with

TGI at the level of 12.0%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

Multidrug-resistant human uterine sarcoma model On day 0 mice Crl:SHO-Prkdc^{scid}Hr^{hr} were grafted subcutaneously (sc) in the right side with 7x10^6 of MES-SA/Dx5 cells suspended in 0.1 ml of HBSS:Matrigel 10:1 mixture by means of a syringe with a 0.5 x25 mm needle (Bogmark). When tumors reached the size of ~ 167-190 mm^3 (day 19), mice were randomized to obtain the average size of tumors in the group of ~ 180 mm^3 and assigned to treatment groups. The treatment groups were administered with the preparations of fusion proteins of the invention of Ex. 6 , Ex.19 (30 mg/kg) and rhTRAIL114-281 (10 mg/kg) as a comparison against formulation buffer (5 mM NaH_2PO_4, 95 mM Na_2HPO_4, 200 mM NaCl, 5 mM glutatione, 0.1 mM $ZnCl_2$, 10% glycerol, 80 mM saccharose, pH 8.0) as a control. The preparations were administered intravenously (*i.v.*) six times every second day. When a therapeutic group reached the average tumor size of ~ 1000 mm^3, mice were sacrificed by disruption of the spinal cord. The control group received rhTRAIL114-281.

The experimental results obtained in mice Crl:SHO-Prkdc^{scid}Hr^{hr} burdened with MES-SA/Dx5 uterine sarcoma treated with fusion proteins of the invention of Ex. 18, Ex. 6 , Ex.19 and comparatively with rhTRAIL114-281 are shown in Fig. 34 as a diagram of changes of the tumor volume and in Fig. 35 which shows tumor growth inhibition (%TGI) as the percentage of control.

The results of experiments presented in the graphs in Figures 34 and 35 show that administration of the fusion proteins of the invention Ex. 6 , Ex.19 caused tumor MES-SA/Dx5 growth inhibition, with TGI respectively 99.7% and 99.7% relative to the control on 33th day of the experiment. For rhTRAIL114-281 used as the comparative reference, the slight inhibitory effect on tumor cell growth was obtained relative to the control, with TGI at the level of 29%. Thus, fusion proteins of the invention exert much stronger effect compared to rhTRAIL114-281 alone.

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.

Claims

1. A fusion protein comprising:

- domain (a) comprising a functional fragment of soluble hTRAIL protein sequence starting with amino acid in a position no lower than hTRAIL95 or a homolog of said functional fragment having at least 70% sequence identity; and

- domain (b) constituting a sequence of anti-angiogenic effector peptide,

wherein the sequence of domain (b) is attached at C - terminus and/or N - terminus of domain (a), and wherein effector peptide is not selected from the group consisting of calreticulin, tumstatin 183-230, kininogen D5, vasostatin, kininostatin and canstatin.

2. A fusion protein according to claim 1, wherein domain (a) comprises a fragment of soluble hTRAIL (SEQ. No. 16) protein sequence starting with amino acid in a range from hTRAIL95 to hTRAIL121, inclusive, and ending with amino acid 281.

3. A fusion protein according to claim 1 or 2, wherein domain (a) is selected form the group consisting of hTRAIL95-281, hTRAIL119-281, hTRAIL120-281 and hTRAIL121-281.

4. A fusion protein according to claims 1 to 3, wherein domain (b) is selected form the group consisting of:

- inhibitors of receptors for growth factors selected from receptors for VEGF, PDGF and EGF;

- fragments of tumstatin, and

- inhibitors of aminopeptidase N (CD13).

5. A fusion protein according to claims 1 do 4, wherein domain (b) consisting of the group of inhibitors of growth factors is selected from VEGF, PDGF and EGF and consist of SEQ. No. 17, SEQ. No. 22 and SEQ. No. 23.

6. A fusion protein according to claims 1 do 4, wherein domain (b) consisting of tumstatin or fragments thereof consist of SEQ. No 18 and SEQ. No. 19 representing respectively tumstatin fragment I and tumstatin fragment II .

7. A fusion protein according to claims 1 do 4, wherein domain (b) consisting of inhibitors of aminopeptidase N (CD13) consist of SEQ. No. 20 and SEQ. No. 21.

8. A fusion protein according to any of the claims 1 to 7, wherein the fusion

protein between the domain (a) and domain (b) contains domain (c) comprising a protease cleavage site, selected from a sequence recognized by metalloprotease MMP, a sequence recognized by urokinase uPA, and combinations thereof.

9. A fusion protein according to claim 8, wherein the sequence recognized by metalloprotease MMP is SEQ. No. 24, SEQ. No. 55 or SEQ. No.56, the sequence recognized by urokinase uPA is SEQ. No. 25.

10. A fusion protein according to claims 8 or 9, wherein domain (c) is a combination of located next to each other sequences recognized by metalloprotease MMP and urokinase uPA.

11. A fusion protein according to any of the preceding claims, wherein the protein between domains (a), (b), (c) and/or (d) comprises additionally glycine, glycine-serine or cysteine flexible steric linker or combinations thereof

12. A fusion protein according to claim 11, wherein flexible steric linker is selected from the group consisting of GG, E, GGGCAAACAAC (SEQ. No. 26), GGCAAACAAC (SEQ. No. 27), GGGGG (SEQ. No. 28), GGGG (SEQ. No. 29), GGG (SEQ. No. 30) and GSG (SEQ. No. 54).

13. A fusion protein according to claim 1, consisting of the amino acid sequence corresponding to the sequence selected form 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.46 ; SEQ. No.47 ; SEQ. No.48 and SEQ. No.49 .

14. A fusion protein according to claim 1, consisting of the amino acid sequence corresponding to the sequence selected form the group consisting of SEQ. No.1 ; SEQ. No.2 ; SEQ. No.3 ; SEQ. No.4 ; SEQ. No.5 ; SEQ. No.6 ; SEQ. No.9 ; SEQ. No.10 ; SEQ. No.11 ; SEQ. No.14 ; SEQ. No.15 ; SEQ. No.46 ; No.47 ; SEQ. No.48 and SEQ. No.49 .

15. A fusion protein according to claim 1, consisting of the amino acid sequence corresponding to the sequence selected form the group consisting of SEQ. No.7 and SEQ. No.8 .

16. A fusion protein according to claim 1, consisting of the amino acid sequence corresponding to the sequence selected form the group consisting of SEQ. No.12 and SEQ. No.13 .

17. A fusion protein according to any of the preceding claims, which is a recombinant protein.

18. A polynucleotide sequence, coding the fusion protein defined as in any preceding claims 1 to 16.

19. A sequence according to claim 18, optimized for genetic expression in *E. coli*.

20. A sequence according to claim 19, selected from the group consisting of SEQ. 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 ; SEQ. No.40 ; SEQ. No.41 ; SEQ. No.42 ; SEQ. No.43 ; SEQ. No.44 ; SEQ. No.45 ; SEQ. No.50 ; SEQ. No.513 ; SEQ. No.52 and SEQ. No.53 .

21. A sequence according to claim 19, selected from the group consisting of SEQ. No.31 ; SEQ. No.32 ; SEQ. No.33 ; SEQ. No.34 ; SEQ. No.35 ; SEQ. No.36 ; SEQ. No.39 ; SEQ. No.40 ; SEQ. No.41 ; SEQ. No.44 ; SEQ. No.45 ; SEQ. No.5051 ; SEQ. No.51 ; SEQ. No.52 and SEQ. No.53 .

22. A sequence according to claim 19, selected from the group consisting of No.37 and SEQ. No.38 .

23. A sequence according to claim 19, selected from the group consisting of SEQ. No.42 and SEQ. No.43 .

24. An expression vector, comprising polynucleotide sequence according to any of the claims 18 to 23.

25. A host cell, comprising the expression vector as defined in claim 24.

26. A host cell according to claim. 25, which is a *E. coli* cell.

27. A pharmaceutical composition, comprising as an active ingredient the fusion protein as defined in any of the preceding claims 1 to 17, in combination with any pharmaceutically acceptable carrier.

28. A pharmaceutical composition according to claim 27, in a form for parenteral administration.

29. Use of a fusion protein as defined in any of the preceding claims 1 to 17 for treatment of neoplastic diseases in mammals, including humans.

30. A method of treatment of cancer diseases in mammal, including human, in a need thereof, comprising administration to a subject an antineoplastic-effective amount of a fusion protein as defined in claims 27 or 28.