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Twelve-membered peptide Tag7 (PGLYRP1) forms a cytotoxic complex with Mt1 (S100A4) and induces cell death via the TNFR1 receptor

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Abstract: The identification of new ligands for proinflammatory receptors expands the understanding of the mechanisms regulating the immune response. Here, we have shown that peptide 17.1—a functional fragment of the protein PGLYRP1 (Tag 7)—forms a stable complex with the full-sized S100A4 (Mts1) protein. The dissociation constant of this complex was $1.28*10^{-8}$M, which indicated a strong binding of the components of this complex. The 17.1-Mts1 complex is capable of causing the death of tumor cells carrying TNFR1, as well as the TNF cytokine and cytotoxic complexes Tag7-Hsp70 and 17.1-Hsp70. The 17.1-Mts1 complex interacts with TNFR1 and induces alternative cytotoxic processes in tumor cells—apoptosis and necroptosis. A shortened fragment of peptide 17.1 interacting with Mts1 and providing the cytotoxic activity of this complex was identified. Thus, a new cytotoxic complex was created and induced the death of tumor cells via the TNFR1 receptor.

Keywords: PGLYRP1, S100A4, TNFR1, cytotoxicity, tumor cells, apoptosis, necroptosis, short peptides.

1. Introduction

Understanding the interaction mechanisms of cytokines and their analogues with specific receptors is the basis of immunotherapy. The binding of cytokines to receptors on the cell surface induces many cellular functions that are often diametrically opposed [1]. A striking example of this is TNFR1, which, depending on the activation of ubiquitinase, can activate intracellular processes, leading to both the proliferation of immune or tumor cells and their death [2].

Accordingly, the specific ligand of this TNF receptor is a key cytokine in the development of the immune system and the pro-inflammatory immune response, and it has the opposite effect on cells and the vital activity of the body [3]. By inducing cell death, TNF can act as an antitumor agent [4]. At the same time, cell death under the action of this cytokine may be one of the causes of the destruction of cartilage and bone tissues during the development of autoimmune arthritis [5]. The expansion of the spectrum of TNFR1 ligands and the understanding of the mechanisms of their action will allow us to outline new approaches to the creation of antitumor and anti-inflammatory therapeutic drugs.

We investigated developing cytotoxic processes during TNFR1 activation and found two new proteins interacting with this receptor: the immune system regulator PGLYRP1 (Tag7) and DNA-hydrolyzing autoantibodies [6,7]. Autoantibodies fully simulated the cytotoxic effect of TNF: They bound to TNFR1 and induced cell death [6].

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Tag7, as well as TNF, interacted with its TNFR1 receptor but did not activate the intracellular processes of cell death. [7]

The ability of Tag7 to form stable complexes with other proteins expands the range of its functions. We have shown that the Tag7-Hsp70 complex has a cytotoxic effect on tumor cells as well as TNF [8]. The bifunctional nature of Tag7 is manifested here. It is able to bind to TNFR1, inhibiting the cytotoxic activity of TNF, and with the help of Hsp70, it participates in the induction of cell death. [6]

We also showed that Tag7 interacts with Mts1 (S100A4), a member of the S-100 family involved in metastasis processes [9]. Both proteins form the Tag7-Mts1 chemotactic complex. This complex has no cytotoxic activity, and it induces the movement of lymphocytes along the concentration gradient of this complex [10]. Interestingly, Mts1 and Hsp70 compete for binding to Tag7. Hsp70 inhibited the movement of cytotoxic lymphocytes, and Mts1 blocked cell death under the action of the Tag7-Hsp70 complex [10]. These results suggest that both proteins bind to the same site of the Tag7 molecule.

Structural and functional analyses of Tag7 made it possible to localize the epitope of this protein necessary for interaction with TNFR1 and Hsp70 [11]. Peptide 17.1 corresponds to the C-terminal section of the chain (aa 163-175); moreover, as a full-sized protein, it inhibits the cytotoxic effect of TNFR1 ligands. In combination with Hsp70, it induces the death of tumor cells [11]. It was also possible to separate the activities of this bifunctional peptide. Two fragments were identified: a fragment responsible for binding to TNFR1 and inhibiting cytotoxicity and a peptide fragment with high affinity for Hsp70 that is involved in the killing of tumor cells [12].

The purpose of this work is as follows: 1) elucidation of the ability of peptide 17.1 to interact with Mts1 and form a cytotoxic complex; 2) characterization of cytotoxic processes induced by this complex; 3) identification of the Mts1 peptide fragment responsible for the formation of the 17.1-Mts1 complex.

2. Results

2.1. Peptide 17.1 binds to Mts1.

In the previous section, we noted the competition between Hsp70 and Mts1 for the binding center with Tag7. We have shown that Hsp70 interacts with peptide 17.1, which is located in the C-terminal region of polypeptide chain Tag7. Here, we investigated the interaction of 17.1 with Mts1 using affinity chromatography. The Mts1 sample was applied to CNBr-activated Sepharose with immobilized peptide 17.1. The bound proteins were eluted and analyzed using SDS-PAGE and a subsequent Western blot with specific anti-Mts1 antibodies. It was shown that Mts1 is present in the eluate (Figure 1). Thus, we can assume that 17.1 binds to Mts.

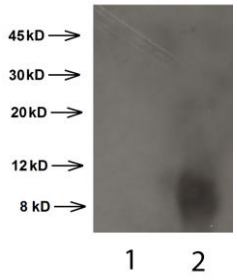

Figure 1. The Mts1 protein was applied to pure Sepharose (1) or peptide 17.1 conjugated with Sepharose (2), eluted with acetonitrile, and resolved using SDS PAGE and Western blot with antibodies relative to Mts1.

2.2 17.1-Mts1 complex has cytotoxic effects on TNFR1-positive tumor cells

Next, we tested the cytotoxic activity of the 17.1-Mts1 complex. The results in Figure 2A indicate that this complex has cytotoxic activity. In addition to the cytotoxic 17.1-Hsp70 complex, the 17.1-Mts1 complex induces the death of the mouse cells of the L929 line and the human leukemia cells of the HL-60 line. Erythroleukemia cells K562 were resistant to the action of this complex; moreover, it was shown earlier for the Tag-Hsp70 cytotoxic complex [13]. Antibodies to TNFR-1 completely block cytotoxicity. Consequently, this receptor is involved in the transmission of a cytotoxic signal.

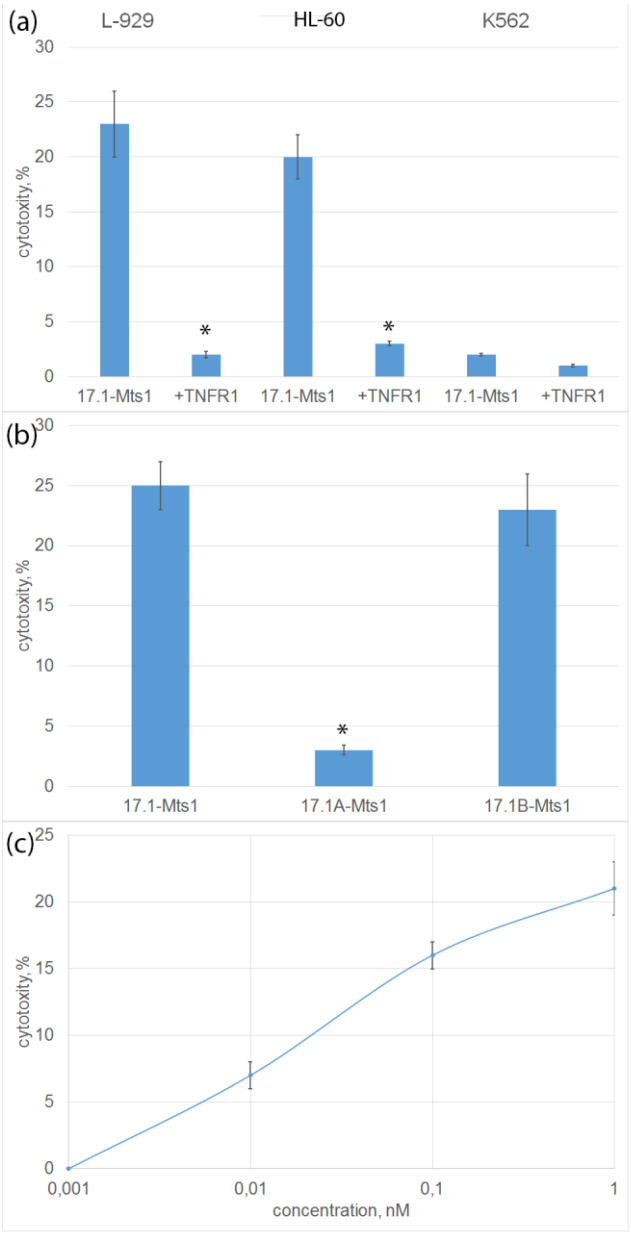

Figure 2(a) Cytotoxic activity of the 17.1-Mts1 complex on L929, K562, and HL-60 cells in the presence of an antibody to TNFR1 (1:100, 24 hours). **(b)** Cytotoxic activity of Mts1 complexes with peptides 17.1, 17.1A, and 17.1B on L929 cells (24 hours). **(c)** Concentration dependence of the cytotoxic activity of the 17.1-Mts1 complex on L929 cells (24 hours). n = 5 for each point (* p-value < 0.05).

As mentioned above, we were able to identify the fragments of peptide 17.1 responsible for binding to TNFR1 and inhibiting the cytotoxic activity of its ligands (17.1A) or

for binding to Hsp70 and inducing cytotoxicity (17.1B). It can be seen (Figure 2B) that in a complex with peptide 17.1A, Mts1 does not contribute to the appearance of cytotoxicity. However, similarly to the 17.1B-Hsp70 complex, the 17.1B-Mts1 complex exhibits cytotoxic activity.

The dependence of cytotoxic activity on the concentration of the 17.1-Mts1 complex was dose-dependent. The maximum value of cytotoxicity is achieved at a concentration of 1nM relative to the 17.1-Mts1 complex.

It should be noted that, unlike the 17.1-Mts1 complex, the complex of this protein with a full-sized Tag7 does not have a cytotoxic effect on cells. To understand the reasons for this difference, a quantitative assessment of the interaction of Mts1 with Tag7 and its peptide fragments was undertaken.

2.3. Peptides 17.1 and 17.1B have a high affinity for Mts1

To evaluate the protein–protein interaction of Mts1 with Tag7 and with peptides 17.1, 17.1 A, and 17.1B, a quantitative microscale thermophoresis method was used to reliably determine the parameters of the interaction of protein complexes [14-16]. Thermophoretic signals obtained by adding peptides and Tag7 to labeled Mts1 demonstrate clear binding curves with an increase in the concentration of studied compounds (Figures 3A-3D). The obtained dissociation constants (K_D) are presented in Table 1.

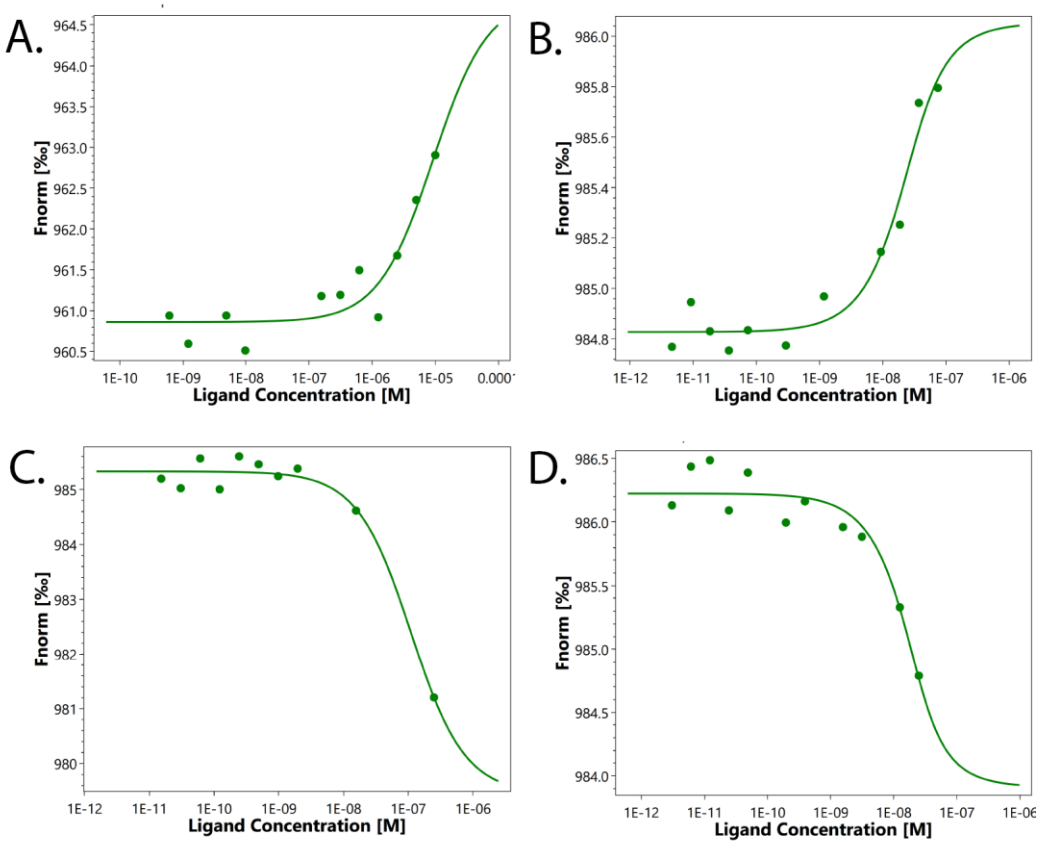

Figure 3 Microscale thermophoresis data for the interaction of Mts1 with Tag7 **(a)** and peptides 17.1 **(b)**, 17.1A **(c),** and 17.1B **(d)**. Each experiment was carried out in triplicate, and the most common data are shown.

Table 1. Apparent dissociation constants obtained via microscale thermophoresis.

Ligands	K_d, nM
Tag7-Mts1	9210 ± 1500
17.1-Mts1	12,9 ± 3
17.1A-Mts1	109 ± 35
17.1B-Mts1	7,26 ± 0.9

It can be observed that the interaction of Mts1 with peptides 17.1 and 17.1B is highly specific. The corresponding binding curves show the nanomolar values of the apparent K_d. It can be assumed that Mts1 forms a strong complex capable of inducing cytotoxicity with these peptides. Mts1 complexes with full-sized Tag7 and peptide 17.1A show a significantly higher apparent K_d, which indicates a lower affinity of these compounds. Apparently, Tag7 and its peptide fragment 17.1A cannot form a stable complex with Mts1, activating the cytotoxic signal via the TNFR1 receptor.

2.4. Mts1 interacts with TNFR1 on the cell's surface

We investigated the ability of peptide 17.1 to form a complex with Mts1 and TNFR1 on the cell surface. The 17.1-Mts1 complex was added to HEK293T cells with TNFR1 on their surface [17]. After incubation, a crosslinking agent (BS^3) was added, forming covalent bonds between molecules located at a distance of less than 1 nm at the time of addition. Next, the cells were lysed, and membrane proteins were precipitated using anti-TNFR1 antibodies conjugated with magnetic beads. The obtained material was separated using SDS electrophoresis and detected using Western blotting with polyclonal antibodies to Mts1 or Tag7. Figure 4 shows that in both cases, products with a molecular weight of about 66 kd are detected, which corresponds to the sum of the masses of TNFR1(55 kD), Mts1 (10 kD), and peptide 17.1 (1.5kD). These results indicate that 17.1-Mts1 interacts with TNFR1 on the surface of HEK293T cells.

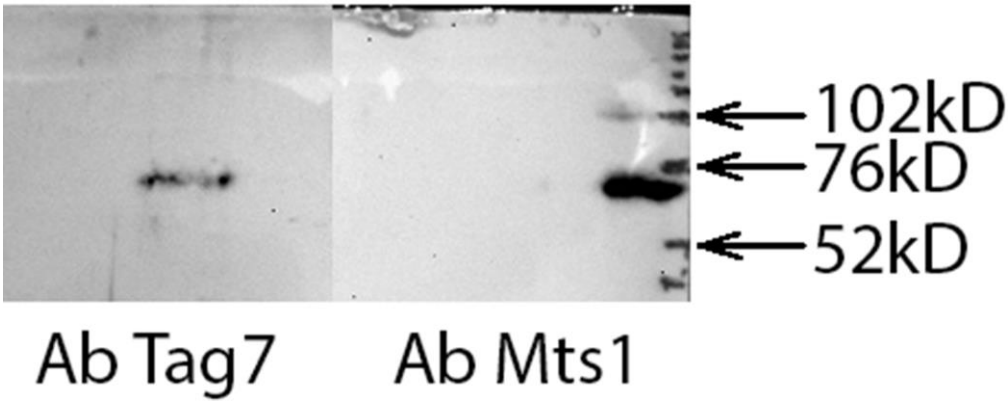

Figure 4 The 17.1-Mts1 complex was added to HEK293T cells and cross-linked with BS^3 with surface proteins; then, the cells were lysed, the cross-linked material was purified on magnetic beads with antibodies relative to TNFR1, and after SDS PAGE and Western blot, the material was stained with antibodies relative to Tag7 (left) or Mts1 (left).

2.5. 17.1-Mts1 complex induces apoptosis and necroptosis in tumor cells

It is known that the TNFR1 receptor induces alternative processes that develop at different time intervals: apoptosis after 3 hours and necroptosis after 20 hours of TNFR1 ligand interaction with the cell [6, 18]. We have previously shown that the cytotoxic com-

plex 17.1-Hsp70 induces both apoptosis and necroptosis. Here, we have shown that cytotoxic processes in tumor cells activated by the action of 17.1-Hsp70 and 17.1-Mts1 complexes are identical. It can be seen that after 3 hours of interaction with the 17.1-Mts1 complex, caspase-dependent apoptosis develops in cells. Cell death was almost completely blocked by caspase inhibitors (Figure 5A). After 20 hours, cell death was prevented in the presence of necrostatin1, an inhibitor of RIP1 kinase, which inhibits necroptotic processes (Figure 5B).

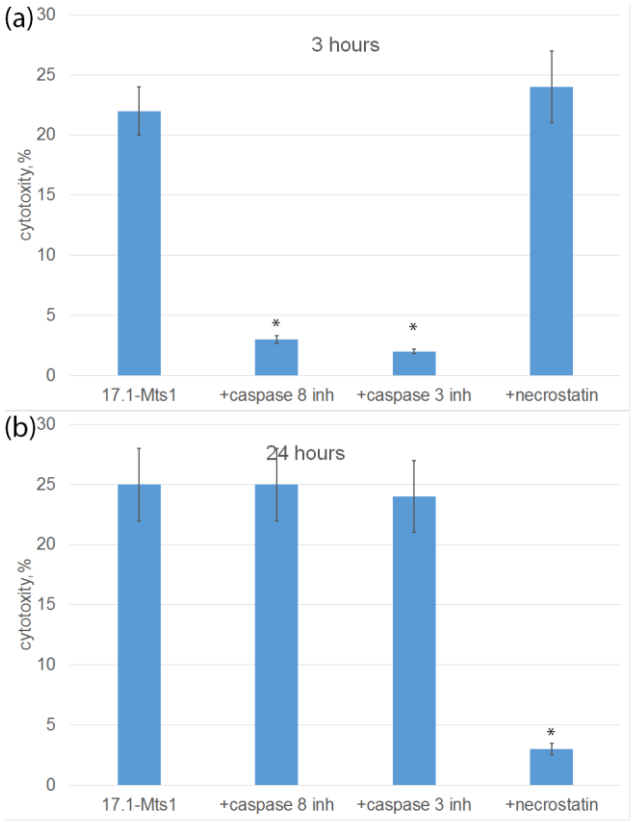

Figure 5. Cytotoxic activity of the 17.1-Mts1 complex on L929 cells after 3 **(a)** and 24 **(b)** hours in the presence of caspase 3 and 8 inhibitors and necrostatin1. n = 5 for each point, (* p-value < 0.05).

2.6. Peptide 17.1 forms a cytotoxic complex with Mts1 peptide fragments

Next, we localized the Mts1 peptide site responsible for the formation of the cytotoxic complex. Mts1 was subjected to limited tryptic hydrolysis to obtain peptides with a molecular weight of 1-2 kg, which is optimal for analysis and subsequent amino acid synthesis. The gel filtration of four-hour hydrolysis products on the Superdex Peptide column revealed several fractions with a molecular weight of 1-3 kDa.

To identify the active fragment, peptide 17.1 was incubated with an aliquot of each fraction, and cell death was tested under the action of the resulting mixture of peptides. The results are presented in Table 2. It can be observed that peptide 17.1 exhibited cytotoxic activity only in a mixture with peptides of fraction No. 7. MALDI analyses of this fraction revealed several peptides that are present in the central region of the Mts1 polypeptide chain.

The results in Figure 6 indicate that this complex, as well as the 17.1-Hsp70 and 17.1-Mts1 complexes, induce cell death via the TNFR1 receptor. Moreover, it can be seen that caspase inhibitors at the 3-hour time point and necrostatin1 at 20 h inhibited the cytotoxic

activity of 17.1 with peptides of the seventh fraction of Mts1 hydrolysis. Thus, this 17.1-N7 complex also induces apoptosis and necroptosis in tumor cells as well as the 17.1-Mts1 complex.

Thus, Mts1 peptide fragments fully simulate the ability of a full-sized molecule to form a cytotoxic complex with a Tag7 fragment.

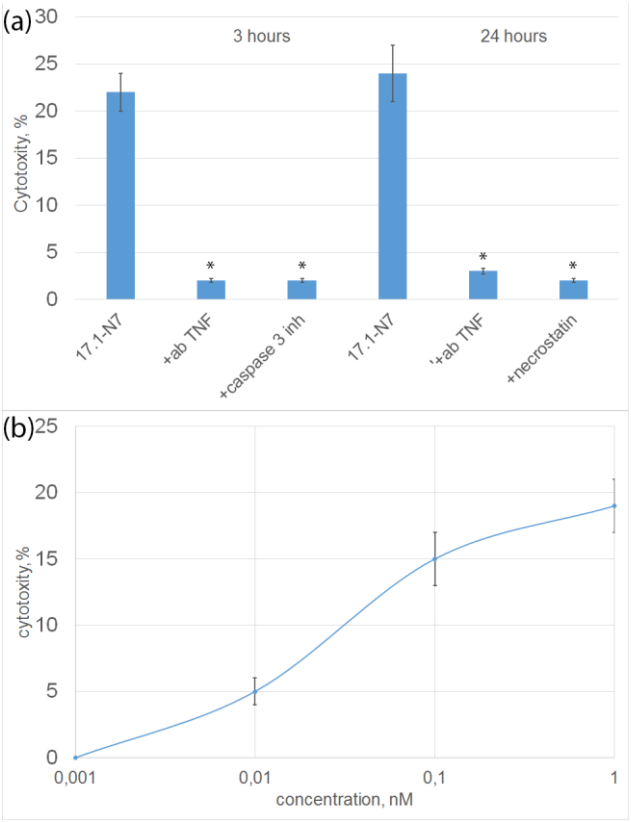

Figure 6. (a) Cytotoxic activity of a mixture of peptide 17.1 with peptides of the 7th fraction of Mts1 hydrolysis on L929 cells after 3 and 24 hours in the presence of caspase 3 inhibitors and necrostatin1. **(b)** Concentration dependence of the cytotoxic activity of mixture 17.1 with peptides of the 7th fraction of Mts1 hydrolysis on L929 cells (24 hours). N = 5 for each point (* p-value < 0.05).

Table 2. Cytotoxic activity of a mixture of peptide 17.1 with Mts1 hydrolysis fractions separated by gel filtration on a Superdex Peptide column.

Fraction number	Cytotoxicity,%	Fraction number	Cytotoxicity,%	Fraction number	Cytotoxicity,%
1	17±3	7	21±4	13	1±0,2
2	6±1	8	3±0,4	14	1±0,3
3	1±0,1	9	1±0,1	15	1±0,4
4	1±0,1	10	1±0,1	16	2±0,2
5	1±0,1	11	1±0,3	17	1±0,3
6	5±1	12	1±0,2	18	5±1

3. Discussion

An interesting result of this work was the production of a new cytotoxic complex consisting of a functional fragment of the Tag7 protein (PGLYRP1) and a full-sized Mts1 protein (S100A4). This complex is capable of causing the cell death of TNFR1-carrying

tumor cells. Similarly to the TNF cytokine and Tag7-Hsp70 or 17.1-Hsp70 cytotoxic complexes, the 17.1-Mts1 complex interacts with the TNFR1 receptor and induces alternative cytotoxic processes in tumor cells: apoptosis and necroptosis. K_d 17.1- Mts1 of the complex was 12,9nM, and it was comparable to the K_d 17.1-Hsp70 complex (4nM), which was obtained by us in a previous study [12]. A low K_d indicates the high affinity of the components of this cytotoxic complex and its stability.

As mentioned above, Tag7 can change its functions. By binding to TNFR1, it can prevent the interaction of this receptor with other ligands and act as an inhibitor. In complex with Hsp70, it reverses its function and activates cytotoxic processes in TNFR1-carrying tumor cells. Recently, we were able to identify the peptide fragments of Tag7 that were responsible for each function. Here, we have shown that, similarly to Hsp70, Mts1 has a low affinity for peptide 17.1A, which inhibits the cytotoxic activity of TNFR1 ligands. Only the 17.1B-Mts1 complex had cytotoxic activity. All of the above indicates the functional identity of cytotoxic complexes 17.1-Mts1 and 17.1-Hsp70.

Interestingly, the full-sized Tag7 does not form a cytotoxic complex with the Mts1 protein [10]. The K_d of the Tag7-Mts1 complex was 9210nM, which explains the instability of the complex. The K_d of the 17.1- Mts1 complex is significantly lower. It may be easier for the shortened Tag7 fragment to bind to the Mts1 epitope, which ensures the interaction of these proteins.

The obtained results expand the understanding of the functional activity of Mts1. The proteins of the S-100 family attract close attention by participating in metastasis processes [19,20]. We have shown that it can participate in immune and antitumor protection. Mts1, presented on the CD3+CD4+ T-lymphocyte membrane, promotes the interaction of cytotoxic T-lymphocyte with the tumor cell membrane [9, 10]. The Tag7-Mts1 complex induces the chemotaxis of cytotoxic lymphocytes. [21]. Here, we have shown that in the complex with peptide 17.1, Mts1 can activate the TNFR1 receptor and induce the death of tumor cells.

As a result of the limited tryptic hydrolysis of Mts1, we were able to identify the peptide fragments of this protein that were capable of activating TNFR1-dependent cell death in a complex with peptide 17.1. Apparently, these peptides are located in the central part of the Mts1 polypeptide chain. However, the exact localization of this epitope will be established only after the study of the biological activity of synthesized peptides with an amino acid sequence corresponding to the peptides of the central region.

Thus, we have created a new cytotoxic complex that induces the death of tumor cells via the TNFR1 receptor. The results obtained can be used in antitumor therapy.

4. Materials and Methods

4.1. Cell Cultivation and Sorting

K562 and HL-60 cells were cultured in RPMI-1640 with 2 mm L-glutamine and 10% FCS (Invitrogen, Carlsbad, CA, USA). L929 and HEK293T cells were cultured in DMEM with 2 mm L-glutamine and 10% FCS (Invitrogen, Carlsbad, CA, USA). These cell lines were obtained from the cell line collection of the N. N. Blokhin National Medical Research Center of Oncology of the Ministry of Health

4.2. Proteins and Antibodies

Recombinant Mts1 was obtained as described in [6].

4.3 Peptides.

Peptides 17.1, 17.1A, and 17.1B were synthesized as described in [12]

4.4. Affinity Chromatography, Immunoadsorption and Immunoblotting

A column with CNBr-activated Sepharose 4B (GE Healthcare, Chicago, IL, USA) conjugated with 17.1 was prepared as described in accordance with the manufacturer's protocol. The Mts1 protein was loaded into a column with sTNFR1 or Hsp70-Sepharose. The excess material was washed out of the column using PBS containing 0.5 M NaCl and only PBS. The protein was eluted with 0.25 M triethylamine (TEA) at pH 12. The eluted material was resolved using SDS-PAGE and applied to a nitro-cellulose membrane [22]. Polyclonal rabbit antibodies to Mts1 or Tag7 (Abcam, Cambridge, UK; 1:15,000; 1 h) followed by HRP-linked antirabbit antibodies (Abcam, Cambridge, UK; 1:15,000; 1 h) were used for detection. The results were visualized using the ECL Plus kit (GE Healthcare, Chicago, IL, USA) in accordance with the manufacturer's protocol. The chemiluminescence was recorded using iBright (Thermo Fisher Scientific, Boston, MA, USA). HEK293T cells (108 cells) were incubated with the 17.1-Mts1 complex (1 nM) in the presence of BS3 (Thermo Fisher Scientific, Boston, MA, USA), lysed in the RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA), purified using Dynabeads (M-280 sheep an-ti-rabbit IgG; Dynal Biotech ASA, Oslo, Norway), and conjugated with anti-TNFR1 antibodies (Abcam, Cambridge, UK) in accordance with the manufacturer's protocol. This material was resolved into 10% SDS-PAGE, followed by Western blotting using antibodies against Tag7 or Mts1 (Abcam, Cambridge, UK).

4.5. Cytotoxicity Assays

For cytotoxicity tests, target cells were cultured in 96-well plates (6 × 104 cells per well), and cytotoxic complexes were added (1nM). Cytotoxicity was measured after 24 h of incubation. The inhibition test was performed with the caspase 3 inhibitor (N-acetil-asp-glu-val-asp-al, 1nM), caspase 8 inhibitor (N-acetil-ile-glu-thr-asp-al, 1nM), or necrostatin1 (1nM) (Sigma-Aldrich, St. Louis, MO, USA). Cytotoxic activity was determined using the MTT test (Promega, Madison, WI, USA) in accordance with the manufacturer's protocol.

4.6. Microscale Thermophoresis

The purified Mts1 was fluorescently labeled using the Alexa Fluor 633 (Eugene, OR, USA) in accordance with the manufacturer's instructions. Mts1 (200 nM) was incubated for 20 min with each compound in the dark at room temperature at 16 different concentrations obtained via sequential dilution, starting from the highest soluble concentration. The samples were loaded into glass capillaries (Monolith NT Capillaries) and analyzed via thermophoresis using Nano-Temperature Monolith NT 115 apparatus (IR laser power 10%). The signal's quality was monitored using a NanoTemper Monolith device to detect the possible autofluorescence of the ligand, deposition, aggregation, or ligand-induced changes in the photo-bleaching rate. The experiments were carried out in triplicate and processed using affinity analysis software (MO Control v.1.6.1, Nano-Temper).

4.7. Statistical Analysis

The data were analyzed using Statistica 6.1 (StatSoft®) software. The Shapiro–Wilk test was used to confirm the normality of the distribution of the data. The results are presented as an average value ± SD. Statistically significant differences were determined using the t-test. The value of $p < 0.05$ was considered statistically significant.

5. Conclusions

In this study, we have created a new cytotoxic complex 17.1-Mts1 that induces the death of tumor cells via the TNFR1 receptor. It appears that several proteins (Mts1 and Hsp70) that can tightly interact with the fragments of the PGLYRP1 (Tag7) protein can also activate the TNFR1 receptor in a complex with these peptides and induce cell death. The results obtained can be used in antitumor therapy.

Author Contributions: Conceptualization, L.P.S. and D.V.Y.; methodology, D.M.Y. and L.P.S.; software, D.V.Y. and D.M.Y.; validation, L.P.S. and D.V.Y.; formal analysis, L.P.S. and D.V.Y.; investigation, E.A.R., D.M.Y., L.P.S., and D.V.Y.; resources, D.V.Y.; data curation, L.P.S. and D.V.Y.; writing—original draft preparation, L.P.S. and D.V.Y.; writing—review and editing, L.P.S. and D.V.Y.;

supervision, L.P.S. and D.V.Y.; project administration, L.P.S. and D.V.Y.; funding acquisition, L.P.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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