**Structure-based screening reveals a ligand that stabilizes the [2Fe-2S] clusters of human mitoNEET and reduces ovarian cancer cells proliferation**

Henri-Baptiste Marjault 1, Yang-Sung Sohn1, Ke Zuo 1**,** Paolo Carloni2,3,4,5, Ron Mittler6 and Rachel Nechushtai1\*.

1 The Alexander Silberman Institute of Life Science and The Wolfson Centre for Applied Structural Biology, Faculty of Science and Mathematics, The Edmond J. Safra Campus at Givat Ram, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel

2 Department of Physics, RWTH Aachen University, 52074 Aachen, Germany

3 Computational Biomedicine Section, Institute of Advanced Simulation IAS-5 and Institute of Neuroscience and Medicine INM-9, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

4 Computational Biomedicine, Institute of Advanced Simulation IAS-5 and Institute of Neuroscience and Medicine INM-9, For-schungszentrum Jülich GmbH, 52425 Jülich, Germany

5 JARA Institute: Molecular Neuroscience and Imaging, Institute of Neuroscience and Medicine INM-11, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

6 Department of Surgery, University of Missouri School of Medicine, and Interdisciplinary Plant Group, Christopher S. Bond Life Sciences Center, University of Missouri, 1201 Rollins St, Columbia, MO 65211,USA

\*Corresponding author (rachel@huji.ac.mail.il)

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**Abstract:**

Human NEET proteins play an important role in a variety of diseases, including cancer. Using the recently published X-ray structure of the human mNT-M1 complex, we screened a commercial chemical compound library and identified a new human mitoNEET (mNT) binding ligand (NTS-01). Biochemical investigations revealed that NTS-01 specifically binds to the human mNT protein and stabilizes its [2Fe-2S] clusters under oxidative conditions *in vitro*. Treatment of ovarian cancer cells with NTS-01 induces ovarian cancer (SKOV-3) mitochondrial fragmentation (fission) and reduces ovarian cancer cell proliferation in a 2D single-layer cell culture, as well as in a 3D spheroids culture. The NTS-01 molecule represents therefore a new lead compound for further drug design studies attempting to develop efficient treatment against ovarian cancer.

**Introduction:**

NEET proteins belong to a novel class of [2Fe-2S] proteins, present across almost all organisms1-2. The [2Fe-2S] clusters of NEET proteins are coordinated by a 3Cys:1His structure, in which the histidine is exposed on the surface of the NEET protein. This [2Fe-2S] coordination structure provides NEET proteins with a unique [2Fe-2S] cluster lability feature, compared to other iron-sulfur proteins1.

Human NEET proteins are involved in the regulation of several important cellular processes (*e.g.,* apoptosis, autophagy, ferroptosis)3, as well as play a key role in mitochondrial iron, reactive oxygen species (ROS) and calcium homeostasis, and mitochondrial morpho-dynamics4-5 . The human NEET family consists of three proteins; the mitoNEET/mNT (encoded by CISD1), the nutrient-deprivation autophagy factor-1/NAF-1 (encoded by CISD2), and the mitochondrial inner NEET protein/MiNT (encoded by CISD3)1. mNT and NAF-1 are homodimeric membrane-bound proteins anchored on the outer mitochondrial membrane (OMM); NAF-1 is also localized to the Endoplasmic reticulum (ER) membranes and to the ER-mitochondrial associated membranes (MAM) that connect the mitochondria to the ER. The third member of the NEET family, MiNT, is a soluble monomer localized inside the mitochondrial matrix.1 MiNT binds two [2Fe–2S] clusters, while in mNT and NAF-1, each monomer binds one [2Fe–2S] cluster6.

Cancer cells exhibit different pH conditions compare to healthy cells7 and due to their [2Fe-2S] clusters pH dependent lability human NEET proteins are involved in a variety of cancers, and are central to breast cancer proliferation and tumor growth8-9. The lability of NAF-1 clusters was shown to play a key role in the development of triple-negative breast cancer. Darash-Yahana et al.10 showed for the first time that human NAF-1 is essential for the proliferation of breast cancer cells and that overexpressing the NAF-1 H114C mutant (with a 25 times higher stability of the [2Fe-2S] clusters) in breast cancer cells, significantly reduced the growth of xenograft tumors10. The lability of NAF-1 [2Fe-2S] clusters is therefore associated with tumorgenicity, and stabilization of NAF-1 clusters leads to a decrease in tumor growth10.

To date, few compounds targeting the stability of NEET proteins' clusters have been reported. Most of these compounds target mNT and stabilize its [2Fe-2S] clusters11. However, none of these stabilizers showed efficacy against cancer proliferation and/or tumor growth. Most of these [2Fe-2S] stabilizing molecules belong to the TDZ and TZD-like compounds, mainly targeting the peroxisome proliferator-activated receptor-γ (PPAR-γ)12. Molecules that specifically target the [2Fe-2S] clusters of NEET proteins could be highly important in the context of cancer therapy due to the involvement of the NEET proteins cluster stability in different types of cancer3, 13. The expression level of mNT protein was shown to be high in many types of cancers, mainly in epithelial cancers that are known to be "iron addicted,"; *e.g.,* ovarian cancer, which is the fifth leading cause of women's cancer death; to which to date there no effective therapeutic solution to treat this cancer type14.

Here we used a structure-based strategy using our mNT-M1 co-crystal coordinates [Marjault et al, (2022)]15 to define the binding areas of different compounds. A chemical library was screened using a classic virtual screening workflow followed by an induced-fit docking calculation algorithm from which the final selection was made. The new compound, NTS-01, was found to bind to mNT. NTS-01 turns out to be an mNT-[2Fe-2S] clusters' stabilizer. It shows high cytotoxicity to ovarian SKOV-3 cells, inducing mitochondrial fission and reducing ovarian cancer cell proliferation.

**Materials and methods:**

**Docking studies of molecules that bind mNT:**

The human mNT-M1 complex structure was obtained from the Protein Data Bank (<http://www.rcsb.org>, PDB 7P0O)15. Using the Protein Preparation Wizard software from Schrodinger16, missing hydrogens were added and the [2Fe-2S] clusters were generated in their oxidative form (a state in which the cluster is the most labile, and similar to the protein state observed in the crystal)15 at pH 7.0. The structure was then refined at pH 7.0 with a restrain minimization using PROPKA pH: 7.0 to assign the protonation states of each residue (convergence of heavy atoms to RMSD at 0.3 Å), and crystal water molecules beyond 3 Å were removed within the OPLS3e force field.16 The ChembridgeTM library of EXPRESS-Pick Stock (500,000 small molecule screening compounds) was prepared using the Schrodinger ligand preparation product, Ligprep (version 12.4, Schrödinger, LLC, and New York-2). Within an OPLS3e force field, ionization was generated at pH 7.0 (+/- 2.0) using Epik, molecules were desalted, and 32 tautomeric forms were generated.17 The gird on the M1-mNT complex was generated using Receptor grid generation in the Glide application (Glide, version 12.4, Schrödinger, LLC, and New York-2)18. The space search was defined by a cube of 27 Å centered on the known ligand (coordinate X, Y, Z: 8.12, 7.53, -4.36). The Schrodinger protocol for structure-based virtual screening that consists of: 1) The absorption, distribution, metabolism, and excretion parameters were predicted using Qikprop19 and the compounds were prefiltered by Lipinski's rule19. The molecules were also selected within a molecular weight between 300 to 700 Da and a predicted cell permeability score higher than 500 (score based on Ligprep application; 2) Ligand preparation using the Schrodinger ligand preparation product, Ligprep, using the default parameters17; 3) The docking was done using glide by three consecutive precision docking calculation (Glide High Throughput Virtual Screening, Standard Precision, and Extra Precision)18 with a scaling factor of 0.80 and a partial cutoff of 0.15 and the use of Epik state penalties for docking. The docking pose result was then used as input for Induced fit docking using the same grid receptor as described above20. The prime refinement was done following the standard protocol for residues within 5.0 Å of the ligand, and the compounds were re-docked by using extra precision glide docking by adding special recognition terms to identify and reward structural motifs important to binding. All dockings were based on OPLS-3e force field with the [2Fe-2S] cluster in its oxidized form. The final selection of the ligand was then based on the docking pose variation of the compound after redocking, and specific attention was given to its interaction(s) with the following residue: histidine 87, Lysine 55, and the Lys 68 of mNT due to their key role in the stability of the [2Fe-2S] cluster1, 11, 15. The physical assumption used to include the residues H87, K55, K68 of mNT was the following: H87 is a key residue in mNT as it binds one the iron ions. K55 forms a stabilizing interaction (either a salt bridge or a water mediated H-bond depending on the protonation state of H87)1, 21. Finally, both T68 insertion and A68 insertion could accelerate the cluster release in vitro22. This shows that K68 is also an important residue for cluster’s stability.

**Protein purification:**

The soluble part of mNT was expressed in *E. coli* and purified as described in 21. Cells were pelleted after expression was induced using 0.25 mM IPTG, resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, and 10 mM MgCl2). 3–5 mg of DNAse, and 3–5 mg of lysozyme were added together with a protease inhibitor solution containing 200 µM aminocaproic acid, 200 µM benzamidine, and 200 µM PMSF). Then the cells were disrupted with a Microfluidizer® cell disruptor. mNT was purified using Ni-agarose and size exclusion chromatography as described in23.

**Cluster stability assay:**

The stability of the [2Fe–2S] clusters of mNT was measured according to24. mNT [2Fe-2S] cluster stability was determined by monitoring the loss of absorption at 458nm using a Synergy™ H1 plate reader equipped with a temperature control apparatus set to 37 °C. The effect of the NTS-01 molecule was measured by incubating mNT with NTS-01 (1:3 molar ratio). Each curve represents the mean (±SD) of three experimental repeats.

**Kd determination:**

The Kd of the binding of NTS-01 to mNT was determined using the isothermal titration colorimetric (ITC) method with a MICROCAL PEAQ-ITC, Malvern instrument. Analyses were performed with MicroCal PEAQ-ITC Analysis Software. All experiments were performed by injecting 1 μL of NTS-01 molecule at 250µM in 100 mM Tris-HCl pH 8.0, 100 NaCl and 5% DMSO, into a 200 μL sample cell containing 50 µM mNT solubilized in the same buffer (with DMSO) at 25 °C. Forty injections were performed with a spacing of 150s with a reference power of 10 μcal/s. A control experiment was performed by titration of the NTS-01 molecule into the buffer. ITC measurements were fitted to a one-site binding model. The established Kd (±SD) value is the average of three independent repeats.

**Confocal Microscopy:**

A total of 2 × 105 cells were plated in micro-slide four-well glass-bottom plates 2 days before the experiment. The cells were treated with 50 µM of NTS-01 compound. The Cells were incubated with the probe rhodamine B-[(1,10-phenanthrolin-5-yl) aminocarbonyl] benzyl ester (RPA) to enable monitoring of their mitochondria with confocal microscopy (Olympus FV3000. confocal laser-scanning microscope). All obtained images were analyzed with ImageJ software.

**3D-Spheroids' cell studies:**

The effect of NTS-01 on the growth of SKOV-3 ovarian cancer cells' spheroids was evaluated using the IncuCyte Zoom® (Essen Bioscience). SKOV-3 cells were seeded in a 96-well ULA plate (Corning 7007) at a density of 2 x 103 and allowed to culture for an additional three days to form 3D-spheroids. The resulting spheroids were then treated with 10, 20, 50, and 100 µM of NTS-01. Cell images were recorded every two hours and analyzed with the IncuCyte® software.

**Cell toxicity studies:**

To test the cytotoxicity of the NTS-01 compound, 8x103 cells per well were seeded into a 96-well cell culture plate one day before the experiment. The cells were treated with 5, 10, 25, and 50 µM of NTS-01 compound for five days with an everyday dose or one-dose treatment for five days. Cytotoxicity was measured by the addition of PrestoBlue™ Cell viability reagent from Invitrogen (10% vol/vol). After one hour of incubation at 37 °C, the fluorescence was measured in a plate reader (excitation, 530–560 nm; emission, 590 nm).

**IC 50 determination:**

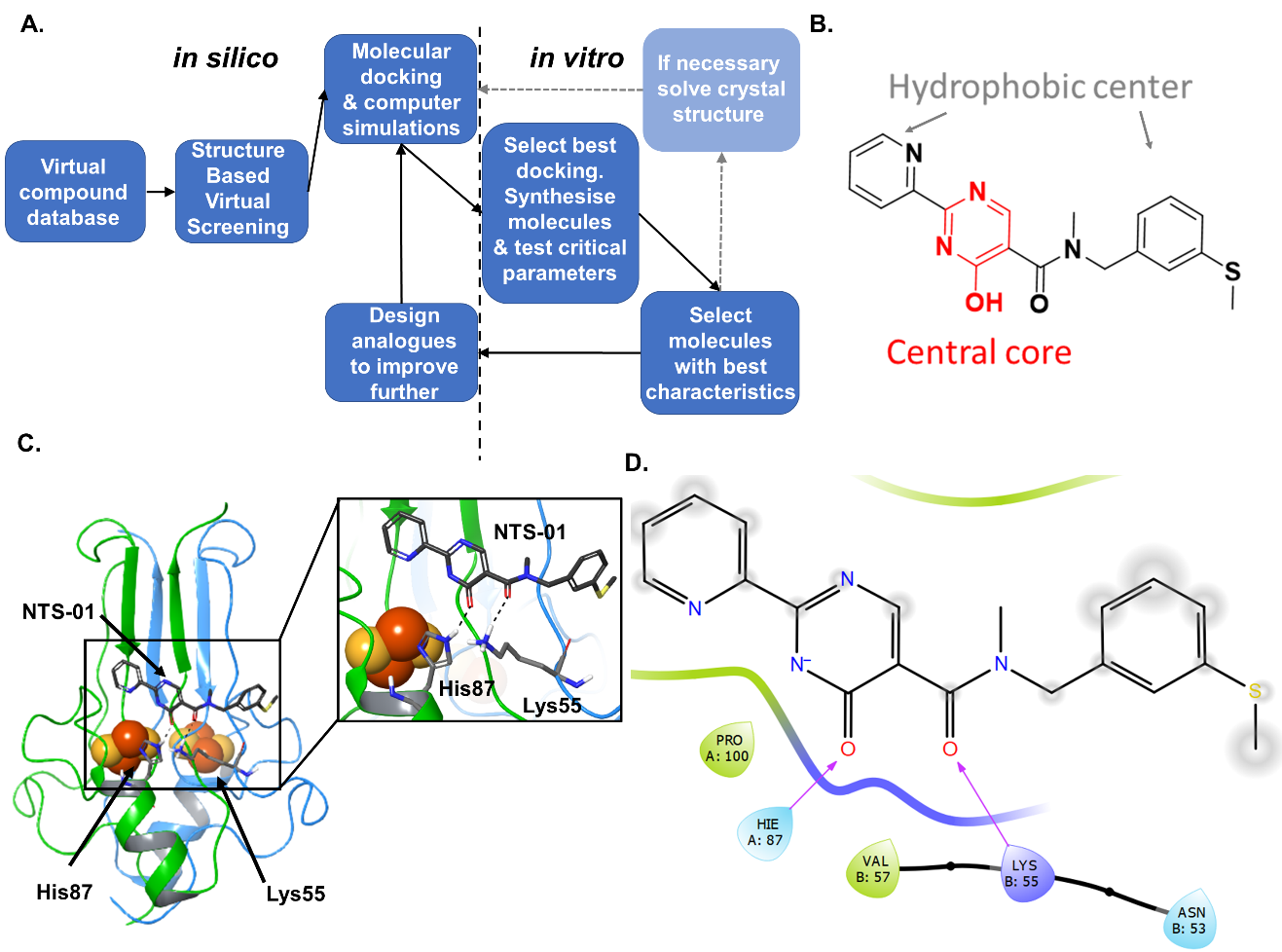
To determine the IC50 of the NTS-01 compound, 8X103 cells were plated into 96-wells plates one day prior to the experiment. The cells were treated with a gradient concentration of NTS-01 from 0.05 to 100 µM. Cytotoxicity was measured using PrestoBlue™ Cell viability reagent from Invitrogen (10% vol/vol), and fluorescence was measured on a plate reader after one h of incubation at 37 °C (excitation, 530–560 nm; emission, 590 nm). The IC50 values were calculated by fitting the plot to the Hill equation:

Y = B + ((T - B) / (1 + 10(log (EC50) - X) × Hill slope)). T and B: top and bottom plateaus of the Y axis.

**Statistical analysis:**

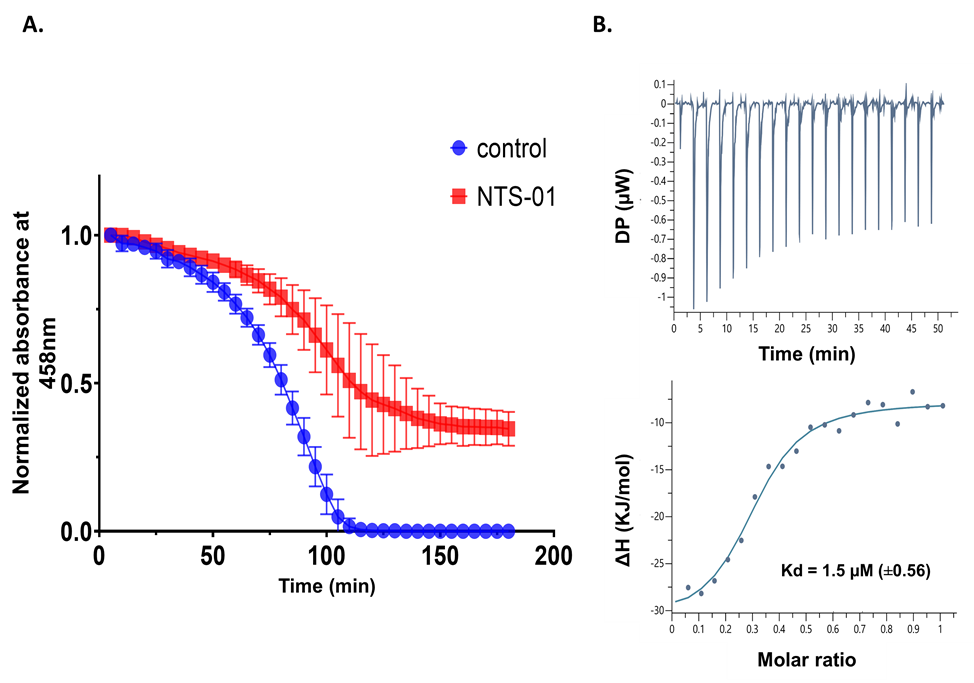
All cell experiments, stability assay, and ITC measurements were repeated at least three times, and the t-test significance of the data was evaluated using the analysis program Prism 8.0.

**Results:**

**Identification of a new compound that stabilizes mNT [2Fe-2S] clusters:**

**Figure 1: Docking, combined with induced fit in silico approach, predicts a novel mNT binding molecule - NTS-01**. **A.** The strategy used in the discovery of the NTS-01 molecule. The association of in-silico and in-vitro methods that screened a library of compounds, identified, and validated a new mNT protein binding molecule. **B.** Structure of the NTS-01 molecule identified. **C.** Docking model of the NTS-01 compound onto the mNT protein. The NTS-01 molecule is predicted to bind at the [2Fe-2S] cluster binding area. **D.** Predicted molecular interaction between the NTS-01 molecule and the mNT protein. NTS-01 is predicted to form a hydrogen bond with histidine 87 and lysine 55 of mNT. The grey atom background represents the solvent-accessible surface area (SASA) of that atom. The hydrophobic moiety of NTS-01 is also predicted to create hydrophobic interactions with the hydrophobic residues of mNT (valine 57, 79 and proline 100).

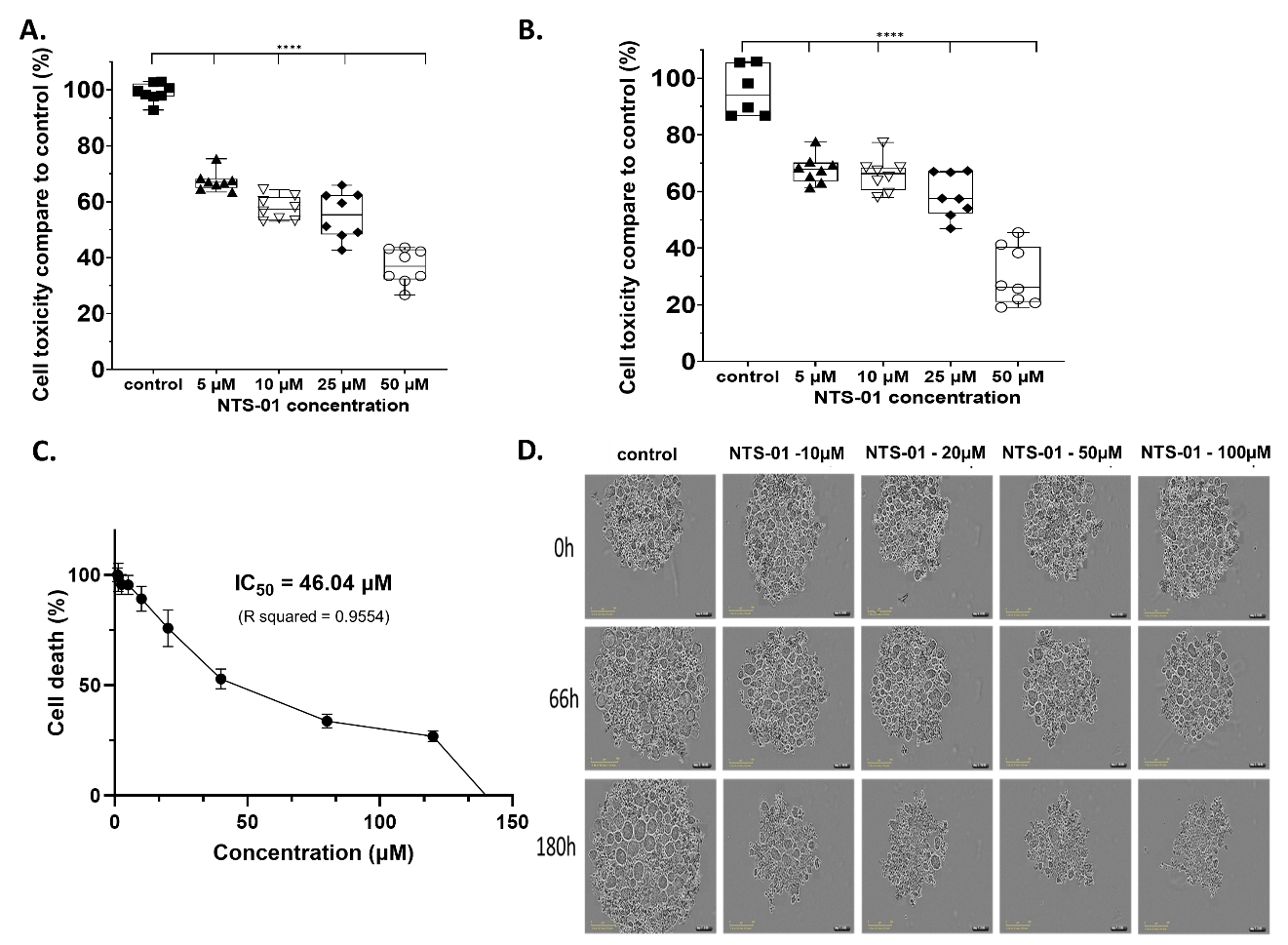
Based on the X-ray pose of M1 on mNT [Marjault *et al*., (2022)]15, we identified a new ligand that binds to mNT (Fig. 1A) by an *in-silico* approach; from a structure-based virtual screening to an induced-fit docking. In the rigid docking, the bond lengths, angles, and torsion angles of the input structures are fixed in the ligand/protein complex generation, only a minimization on structure translation and rotation is applied, while for induced fit docking, conformational changes of the protein side chains induced by ligand binding are considered. The latter can generate a more accurate complex structure and rescue false negatives in virtual screening against a single conformation of the receptor in rigid body docking. Here, a widely used force field in ligand-protein binding prediction, OPLS3e force field14, was used in the screening. Its high level of accuracy in assessing small molecule conformational propensities and solvation as well as ligand-protein interactions is suitable especially for medicinally relevant chemical structures binding to the protein, including ones with shallow binding pocket, like PD-L1 dimer interface25, D3 dopamine receptor26, SARS-CoV-2 main protease27, also the case of mNT protein here. Based on the ranking of binding scores evaluated by the energy-based GlideScore scoring function28, 13 out of 1.2 million structures with the highest score (i.e., the ligands with top 10% lowest binding free energy to mNT) were determined as positive hits for further in vitro experimental tests. Among the positives hits, we identified the ligand 4-hydroxy-N-methyl-N-[3-(methylthio) benzyl]-2-pyridin-2-ylpyrimidine-5-carboxamide (NTS-01). This molecule features a pyrimidin-4-ol at its center with hydrophobic extremity (Figure 1B). The docking calculation predicted that NTS-01 interacts with mNT in the vicinity of its [2Fe-2S] clusters (Fig. 1C & Figure S1). NTS-01 is predicted to form a hydrogen bond with histidine 87 and the lysine 55 (Fig. 1D) of mNT (Fig. 1 and Figure S1) and bind in close vicinity of the [2Fe-2S] cluster domain. Moreover, the induced fit docking calculation showed a flexibility of the Lysine 55 and after redocking NTS-01 was predicted to interact with mitoNEET by forming hydrogen bond with the Lysine 55 and Histidine 87 residue (Table S1D & Figure S1).

**NTS-01 stabilizes the [2Fe-2S] clusters of mNT:**

**Figure 2: NTS-01 binds to mNT and stabilizes its [2Fe-2S] clusters in-vitro.** **A.** Cluster stability assay reveals that NTS-01 increases the stability of the [2Fe-2S] clusters of mNT at pH 6.0 and 37°C. Cluster stability was determined by following the specific absorption peak of mNT bound [2Fe–2S] at 458 nm in the presence (red square) or absence (blue circle) of NTS-01 (60µM); n=3. **B.** Isothermal titration colorimetric (ITC) peak (upper panel) and its fitting model curve (lower panel) showing the binding of mNT (50 µM) to the NTS-01 compound (250 µM) at pH 8.0 and 25 °C; n=3.

To investigate the binding of NTS-01 to mNT experimentally, solubilized NTS-01 and mNT protein were incubated at pH 6.0 and the effect of NTS-01 on mNT cluster stability was tested compared to non-treated mNT (control). After 180 min, up to 35% of the [2Fe-2S] clusters remained bound to mNT compared to control, and the half-time to reach 50 % loss of mNT [2Fe-2S] clusters was shifted from 80 min to 110 min (Fig. 2A). Moreover, in the presence of NTS-01, about ~35% of the clusters remain attached to mNT. The binding affinity of NTS-01 to mNT was determined using the isothermal titration colorimetric (ITC) method with the MICROCAL PEAQ-ITC. Due the high lability of mNT [2Fe-2S] clusters at pH 6.021, the measurements were performed at pH 8.0. On this condition, NTS-01 was found to bind to mNT protein with a Kd of 1.5 µM (±0.56) (Fig. 2B).

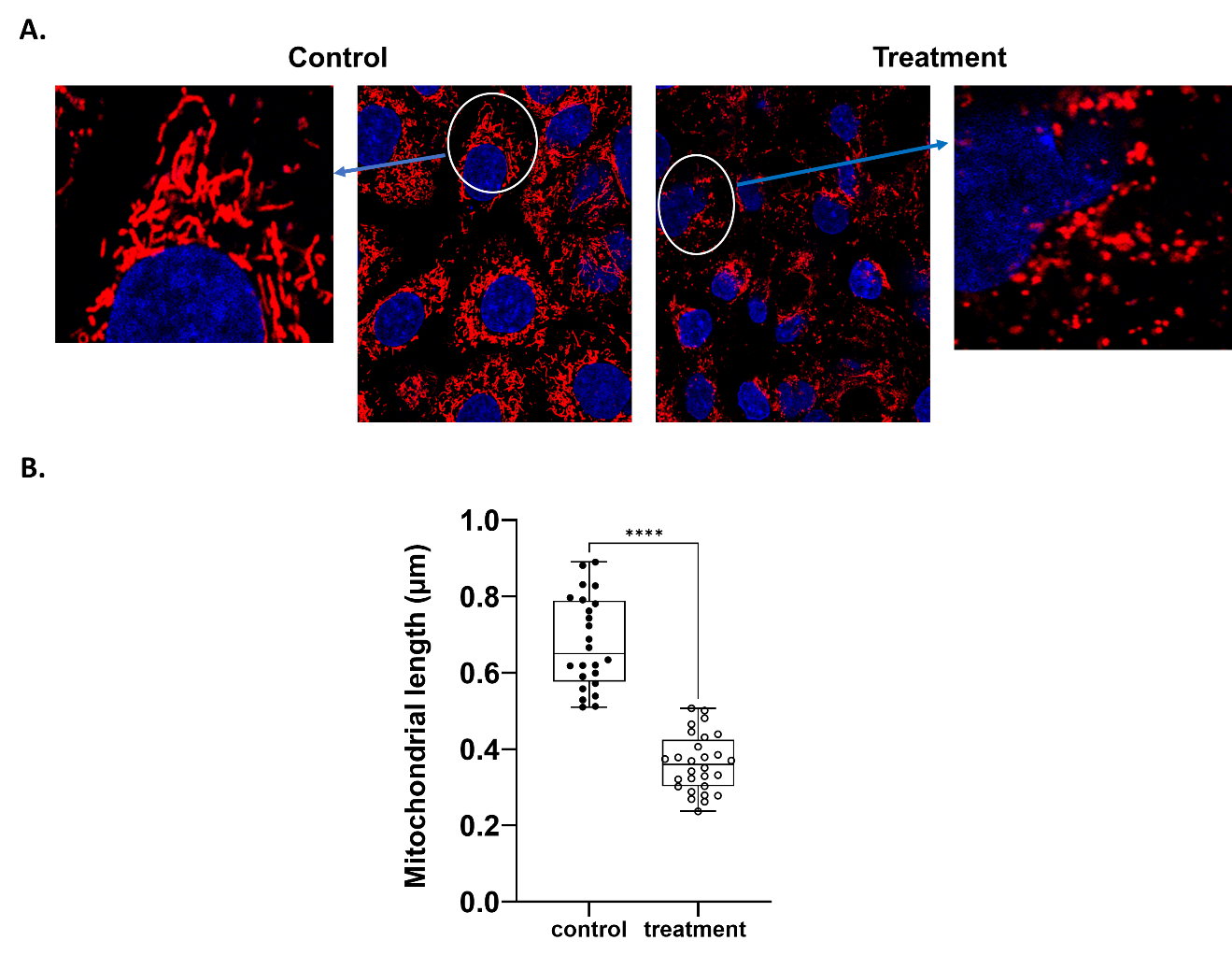
**NTS-01 is cytotoxic to ovarian cancer cells (SKOV-3) causing an inhibition of cell proliferation:**



**Figure 3:** **Cytotoxicity of NTS-01 to SKOV-3 ovarian cancer cells.** **A.** Ovarian cancer SKOV-3 cells were treated with a single dose of 5 µM (filled triangle), 10 µM (empty inverse triangle), 25 µM (black circle), and 50 µM (empty circle) of NTS-01, and the cytotoxicity was measured after five days of incubation; n=3, p<0.00001. **B.** Ovarian cancer cells (SKOV-3) were treated for five days with a daily dose of NTS-01 at a concentration of 5 µM (filled triangle), 10 µM (empty inverse triangle), 25 µM (black circle), and 50 µM (empty circle); n=3, p<0.00001. **C.** IC50 of NTS-01 was determined for SKOV-3 cells by fitting the plot to the Hill equation (see material and methods). **D.** Representative images of SKOV-3 spheroids growth in the presence or absence of NTS-01. Spheroids were treated with 10, 20, 50, and 100 µM of NTS-01 and monitored using the IncuCyte® Zoom system (Essen Bioscience). See supplemental Figure 2 for growth curve.

In order to test the cytotoxicity of NTS-1 to 2D ovarian cancer cell cultures, SKOV-3 cells were treated with 5, 10, 25, and 50 µM of NTS-01 for five days using two different treatments. The first used a single dose at the beginning of the five-day incubation (Fig. 3A), and the second used a daily dose application, *i.e.*, a daily treatment in each of the five days (Fig. 3B). NTS-01 displayed dose-dependent cytotoxicity under both conditions.

Following the observations that the NTS-01 affects SKOV-3 cell viability, we determine the IC50 of NTS-01 for the ovarian cancer cells to be 46 µM (rsquare = 0.9554) (Fig. 3C). To determine the effect of NTS-01 on ovarian cancer cells when cells are in close contact creating a microenvironment known to mimic the condition of a small tumor, we prepared spheroids of SKOV-3 cells, treated them with different concentrations of NTS-01 (10, 20, 50, and 100 µM) and followed their growth over seven days compared to non-treated spheroids (control). NTS-01 was found to hinder spheroids' proliferation even at a low concentration (Figure 3 & Figure S2. The growth of SKOV-3 spheroids was followed by imaging every 2 hours using the Incucyte® system. Treatment of the SKOV-3 spheroids with the NTS-01 molecule showed that ovarian cancer cells' spheroids stopped their proliferation 66 hours after treatment. After 180 hours, the spheroids shrank to an empty-spheroids appearance (Fig. 3D). The growth curve of the spheroid showed that the NTS-01 compound is effective at low concentrations (Figure S2).

**NTS-01 induces mitochondrial fission in ovarian cancer cells:**

**Figure 4: NTS-01 induced mitochondrial fragmentation/fission in ovarian SKOV-3 cancer cells.** **A.** Representative confocal microscopy images of SKOV-3 cells mitochondria, stained with rhodamine B-[(1,10-phenanthrolin-5-yl) aminocarbonyl] benzyl ester (RPA), in the presence (right panel, treated) or absence (left panel, control) of 50 µM of NTS-01. NTS-01 induced mitochondrial fragmentation (enlarged right panel) compared to control cell (enlarged left panel). **B.** Quantification of mitochondrial fragmentation showed that after treatment with 50 µM of NTS-01 the mitochondrial length of treated SKOV-3 cell decreased significantly (n=3, P<0.00001).

Mitochondrial fragmentation/fission was linked to cell death activation/regulation in cancer cells3, 13, 29. Stabilizing the [2Fe-2S] clusters of NEET proteins increased mitochondrial fission in several cell types, *e.g.,* breast cancer cells3, 10, 29. Ovarian cancer cells have high levels of mNT protein30, making them an excellent candidates to test the effect of NTS-01 on mitochondrial fragmentation/fission. Fig.4 shows that treatment of ovarian cancer-SKOV-3 cells with 50 µM of NTS-01 induced mitochondrial fission. Confocal analysis of mitochondrial length showed that ovarian cancer cells treated with 50 µM of NTS-01 for 24 h contain high levels of fragmented mitochondria (Fig. 4A & B).

**Discussion:**

Based on the mNT-M1 X-ray structure (Marjault *et al.*, 2022), we identified *in silico* a new compound that binds to mNT. This compound (NTS-01) differs from the first mNT ligand identified, pioglitazone (PGZ), which acts as a peroxisome proliferator-activated receptor-gamma activator31. Since the identification of PGZ as a stabilizer of mNT [2Fe-2S] clusters, other related compounds were identified, belonging to the thiazolidinedione family (TZD)11. Here we report on a new ligand (NTS-01) that stabilizes the mNT [2Fe-2S] clusters. The NTS-01 compound contains a different central moiety compare to the classical TZD molecules and derivates11. The presence of a pyrimidin-4-ol with two hydrophobic extremities (Figure 1B) could provide NTS-01 with higher solubility, compares to TZDs (Table S1B)1. This higher solubility and structural differences could allow to NTS-01 a better binding affinity compared to other TZD molecules or derivates (Figure 2B) 32-34. The new compound identified does not contain the Tazolidine-2,4-dione (TZD) moiety which is known to activate the peroxisome proliferator-activated receptor-gamma activator.

NEET proteins were shown to be involved in cancer maintenance and proliferation8, 11, 23, 35 via their mitochondrial function, iron/ROS accumulation, and morpho-dynamics. Darash-Yahana *et al. 10* showed an effective approach for reducing cell proliferation and tumor growth by stabilizing the NEET protein NAF-1 [2Fe-2S] clusters.11, 35. Similar to NAF-1, targeting the iron balancing role of the mNT protein could increase the impact of treatments against cancer cells known to be 'iron addicted', like ovarian cancer14, 30. This cancer type, to date, does not have a clinically efficient treatment14, 30. Here we show that NTS-01 specifically stabilizes the [2Fe-2S] clusters of mNT and reduces the cell proliferation of ovarian cancer cells. Moreover, NTS-01 induces mitochondrial fission that probably leads to SKOV-3 highly decreased proliferation, thereby this molecule introduces of a new drug development lead that may contribute to the development of future a therapeutic approach for targeting ovarian cancer. In future studies the effect of NTS-01 on NAF-1 cluster stability should be determined.

**Supporting Information:**

Supporting tables and figures containing additional results.

**Acknowledgement:**

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TOC graphic

Diagram

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