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A protein–small molecule conjugate as a bispecific inhibitor of proteases implicated in cancer metastasis

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Matrix metalloproteinase-9 (MMP-9) and kallikrein 6 (KLK6) are key extracellular proteases implicated in metastatic progression. In colorectal and pancreatic cancers, their overexpression correlates with aggressive disease and poor prognosis, making them attractive therapeutic targets. While monospecific inhibition of either protease shows limited efficacy, simultaneous dual inhibition has not yet been explored as a strategy to block metastasis. To address the lack of an efficient inhibitor of the above metastatic cancers, we prepared a bispecific inhibitor by the site-specific conjugation, via click chemistry, of a selective inhibitor of MMP-9 to a small molecule specifically inhibiting KLK6; the inhibitor comprised the N-terminal domain of tissue inhibitor of metalloproteinases 2 (N-TIMP2), bearing the non-canonical amino acid propargyl lysine (PrK), conjugated to a small molecule known as DKFZ-938. The bispecific inhibitor, thus designated N-TIMP2_{PrK}-DKFZ-938, exhibited inhibitory activity against purified KLK6 and both purified and cellular MMP9 in the absence and presence of KLK6. Functional assays of the bispecific inhibitor in HCT-116, PANC-1, and CAPAN-2 cancer cell lines expressing both MMP-9 and KLK6 demonstrated dose-dependent inhibition of migration and invasion that outperformed the respective monospecific inhibitors, alone or in admixture, without affecting cell viability. These results thus show N-TIMP2_{PrK}-DKFZ-938 to be a promising dual-targeting therapeutic prototype, providing simultaneous inhibition of two proteases that cooperatively drive metastasis.

Keywords Bi-specific inhibitor, TIMP, MMP, KLK, DKFZ-938, Non-canonical amino acids, Click chemistry, Migration, Invasion

Abbreviations

ADC	Antibody-drug conjugate
BGU	Ben-Gurion University of the Negev
Cy5.5-Az	Cyanine5.5 azide
ECM	Extracellular matrix
FBS	Fetal bovine serum
KLK	Kallikrein
MMP	Matrix metalloproteinase
MMP-9 _{CAT}	Catalytic domain of MMP-9
ncAA	Non-canonical amino acid
PrK	Propargyl lysine
TIMP	Tissue inhibitor of matrix metalloproteinase
SD	Standard deviation
SEM	Standard error of the mean

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Pancreatic¹ and colorectal^{2,3} cancers are highly aggressive malignancies with poor prognosis and limited treatment options. In these cancers, membrane-anchored receptors and soluble extracellular proteases act in concert to drive tumor progression, invasion, and metastasis^{4–7}. Their cooperative activity in the tumor microenvironment creates challenges for effective therapy, as inhibition of a single target alone (i.e., monospecific therapy) often fails to fully suppress the cooperative metastatic processes driven by such pairs of molecular targets^{8–12}.

With the aim to overcome the limitations of monospecific therapies for pancreatic and colorectal cancers, research is currently being devoted to developing combination therapies and bispecific inhibitors targeting multiple pathways. For pancreatic cancer, bispecific strategies are showing encouraging preclinical efficacy. For example, a TNF-related apoptosis-inducing ligand (TRAIL) receptor 2/cadherin-3 (TRAILR2/CDH3) bispecific antibody selectively induced apoptosis in CDH3-positive pancreatic cancer cells and drove tumor regression in xenograft models, thus demonstrating superior activity compared to the respective monospecific treatments.⁴ Likewise, a bispecific nanobody (VHH domain) targeting programmed death-ligand 1 (PD-L1) and chemokine receptor type 4 (CXCR4) inhibited pancreatic tumor growth more effectively than either monospecific nanobody targeting its respective antigen separately.⁵ Similarly, an engineered PD-1/IL-2 variant fusion protein, consisting of a PD-1 domain linked to a modified IL-2 cytokine, enhanced T-cell activation, thereby providing an alternative dual-function approach to modulating the immune microenvironment in pancreatic ductal adenocarcinoma.¹³

For metastatic colorectal cancer, dual targeting of Kirsten rat sarcoma (KRAS) G12C and epidermal growth factor receptor (EGFR) with sotorasib and panitumumab improved response rates compared to monotherapy.¹⁴ Bispecific antibody-drug conjugates (ADCs), such as CDH17/GUCY2C or PD-L1/CD3 bispecific antibodies, have also shown promising efficacy; for example, in colorectal cancer models, a CDH17/GUCY2C bispecific ADC conjugated to the ferroptosis inducer RSL3 induced potent cytotoxicity, giving stronger inhibition of tumor growth than the corresponding monospecific ADCs.^{6,7} However, the efficacy of these strategies depends on high and consistent co-expression of both targets, which can vary among patients and during disease progression.^{15,16} In addition, bispecific antibodies targeting membrane receptors may be limited by the requirement for both targets to be situated in close spatial proximity on the cell membrane for simultaneous binding to occur.^{17–19}

To address limitations such as those described above, we developed a bispecific inhibitor capable of simultaneously targeting two key soluble extracellular drivers of metastasis in pancreatic and colorectal cancers, namely, matrix metalloproteinase-9 (MMP-9), a member of the zinc-dependent matrix metalloproteinase family²⁰, and kallikrein 6 (KLK6), a member the kallikrein family of serine proteases. The need for R&D on a bispecific inhibitor derives from the lack of successes in developing inhibitors targeting MMP-9 or KLK6: Attempts to inhibit MMP-9 with monoclonal antibodies, such as batimastat, marimastat, prinomastat, and andecaliximab, have faced challenges such as off-target effects and dose-limiting toxicities^{21–25}, and those to inhibit KLK6 with small molecules or engineered proteins, such as APP-4 M, have not yet progressed to clinical use.²⁶ Our bispecific inhibitor offers a strategy to overcome these limitations by simultaneously targeting MMP-9 and KLK6 in the extracellular environment of cancer cells, thereby enhancing specificity and potency through avidity effects, which may allow lower, less toxic doses. To the best of our knowledge, no prior approach has successfully targeted both MMP-9 and KLK6 in pancreatic or colorectal cancers, highlighting the novelty of this bispecific strategy.

We prepared the bispecific inhibitor by site-specific conjugation via click chemistry, as follows. DKFZ-938, a derivative of the small-molecule KLK6 inhibitor DKFZ-251²⁷ (PMID: 30212625), bearing a pendant azide moiety, was coupled by site-specific conjugation to the N-terminal domain of tissue inhibitor of metalloproteinases 2 (N-TIMP2) that had been modified to bear the non-canonical amino acid (ncAA) propargyl lysine (PrK) in position 31 as a bioorthogonal chemical handle. The resultant conjugate molecule, designated N-TIMP2-PrK-DKFZ-938, comprises, respectively, a selective inhibitor of MMP-9 and a specific inhibitor of KLK6, allowing precise and selective inhibition of these two cooperative extracellular proteases that drive metastasis. In normal healthy tissue, MMP-9 degrades components of the extracellular matrix (ECM), such as collagen IV and V and gelatin²⁸, and it also contributes to tissue remodeling, angiogenesis, and wound repair.^{29–31} KLK6 is involved in normal tissue homeostasis, including skin desquamation and neuronal plasticity.^{32–36} However, in pancreatic and colorectal cancers, both enzymes show markedly elevated expression and activity in tumor tissues and the tumor microenvironment, acting as key drivers of metastasis by promoting ECM degradation and tumor cell invasion and migration.^{37–44} Both proteases are secreted as inactive zymogens and are activated in the ECM, where their activities are closely correlated: in pancreatic and colorectal cancer KLK6 has been shown to enhance MMP-9 activation, and MMP-9 activity is reinforced by KLK6, creating a mutually amplifying interaction that drives pro-metastatic signaling.^{45–49} Targeting the two enzymes simultaneously may thus offer a strategic way to block the cooperative mechanisms that drive metastasis.

Our rationale for selecting N-TIMP2 and DKFZ-938 for engineering the bispecific inhibitor that simultaneously targets the above proteases may be described in brief as follows. TIMPs are small endogenous proteins that regulate MMP activity through high-affinity, non-covalent binding, thereby modulating ECM turnover and tissue remodeling.^{50–52} Among the four human TIMPs, the N-terminal domain of TIMP-2, namely, N-TIMP2, was selected for this study for three reasons: it exhibits potent inhibitory activity against MMP-9 (by coordinating to the catalytic zinc ion); it retains full inhibitory function without being subject to the potential pro-MMP activation mediated by the C-terminal domain^{53–56}; and the N-TIMP2 scaffold is highly amenable to protein engineering, tolerating amino acid substitutions or insertions without a loss of stability or inhibitory activity.⁵⁷ DKFZ-938, the KLK6-targeting component of our conjugate, was chosen for its covalent and selective binding to the catalytic serine residue (Ser195) within the active-site pocket of KLK6, thus mimicking the natural substrate of KLK6.⁵⁸ Our working hypothesis was that the resulting conjugate would enable simultaneous inhibition of MMP-9 and KLK6 in the extracellular environment, effectively targeting the cooperative proteolytic network driving metastasis while maintaining the structural integrity and functional activity of both components.^{46–49}

Our results showed that the bi-specific inhibitor N-TIMP2_{PrK}-DKFZ-938, targeting MMP-9 and KLK6, consistently suppressed – in a dose-dependent manner – migration and invasion of three cancer cell lines, at concentrations as low as the low micromolar range (0.5–1 μ M), with superior efficacy to monospecific inhibitors, each alone and the two in combination. Notably, the inhibition effect was not accompanied by any impairment of cell viability, suggesting that the selective disruption of the extracellular proteolytic network may impede metastatic processes while preserving normal cellular functions.

Results and discussion

Production of N-TIMP2_{PrK}-DKFZ-938

To generate the bispecific protein–small molecule conjugate N-TIMP2_{PrK}-DKFZ-938, we used TIMP2_{PrK}, an N-TIMP2 variant with PrK incorporated into position 31.⁵⁹ For the classical azide-alkyne click reaction used to prepare the conjugate, PrK was selected for its alkyne group, and position S31 was chosen because it is located on an accessible, solvent-exposed, flexible loop of wild-type N-TIMP2 (N-TIMP2_{WT}) that is distant from its site of interaction with MMP-9, thus ensuring minimal interference with MMP-9 inhibition by N-TIMP2_{PrK}-DKFZ-938.^{59,60} To verify successful incorporation of PrK, N-TIMP2_{PrK} was first expressed in *Escherichia coli* strain WK6 on a small scale. Western blot analysis, using an antibody against the C-terminal 6 $\times$ His tag, confirmed the expression of full-length N-TIMP2_{PrK} (15 kDa) in a PrK-dependent manner (Figure S1A). Thereafter, large-scale production and purification of N-TIMP2_{PrK} was undertaken by using affinity chromatography (Figure S1B), followed by SDS-PAGE (Figure S1C) and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis, confirming the expected mass difference of $\sim$ 123.15 Da between N-TIMP2_{WT} and N-TIMP2_{PrK} (Figure S1D).

To identify the optimal conditions for the conjugation of N-TIMP2_{PrK} to the azide-derivatized small molecule DKFZ-938 (whose structure is shown in Figure S2), we first performed a proof-of-concept reaction on a small scale at various N-TIMP2_{PrK}:azide molar ratios, ranging from 1:0.5 to 1:10, where Cyanine5.5 azide (Cy5.5-Az) was used as a mimetic for DKFZ-938 due to its similar molecular weight (715.7 Da) and ease of detection (via a fluorescence signal). A molar ratio of 1:5 resulted in complete conjugation (Figure S3A). A successful conjugation reaction, performed on a large scale with a 1:5 molar ratio of N-TIMP2_{PrK} to azide-DKFZ-938, yielded pure N-TIMP2_{PrK}-DKFZ-938, with a conjugation yield of 93%, as confirmed by SDS-PAGE (Figure S3B) and MALDI-TOF mass spectrometry (Figure S3C).

To test the functionality of N-TIMP2_{PrK}-DKFZ-938, we conducted an assay determining its inhibition of the catalytic domain of MMP-9 (MMP-9_{CAT}). The extent of inhibition of MMP-9_{CAT} followed the order of N-TIMP2_{WT} > N-TIMP2_{PrK} > N-TIMP2_{PrK}-DKFZ-938, with the conjugate having an inhibition constant (K_i) $\sim$ 20-fold higher than for N-TIMP2_{WT} but still in the nanomolar range ($\sim$ 0.68 nM), meaning that the conjugate retained functional inhibitory activity (Fig. 1A; Table 1). Similarly, N-TIMP2_{PrK}-DKFZ-938 inhibited KLK6 enzymatic activity, as evidenced by a half maximal inhibitory concentration (IC_{50}) of 1.0 ± 0.2 μ M after 30 min incubation of KLK6 with the inhibitors (Fig. 1B, C; Table 1). As expected for covalent inhibitors, time-dependent inhibition behavior was observed for DKFZ-938 and N-TIMP2_{PrK}-DKFZ-938, as reflected in their k_{inact}/K_i values, which were $\sim$ 10-fold lower for the conjugate than for DKFZ-938.

The findings that position S31 in N-TIMP2 tolerated substitution with the ncAA and that conjugated N-TIMP2 was still able to maintain substantial inhibitory activity are in agreement with the study of Hayun et al., showing that incorporation of PrK at this position in N-TIMP2 did not impair its binding to MMP-14_{CAT}.⁵⁹ Moreover, the obtained K_i values are consistent with previously reported K_i values for N-TIMP2 mutants inhibiting MMP-

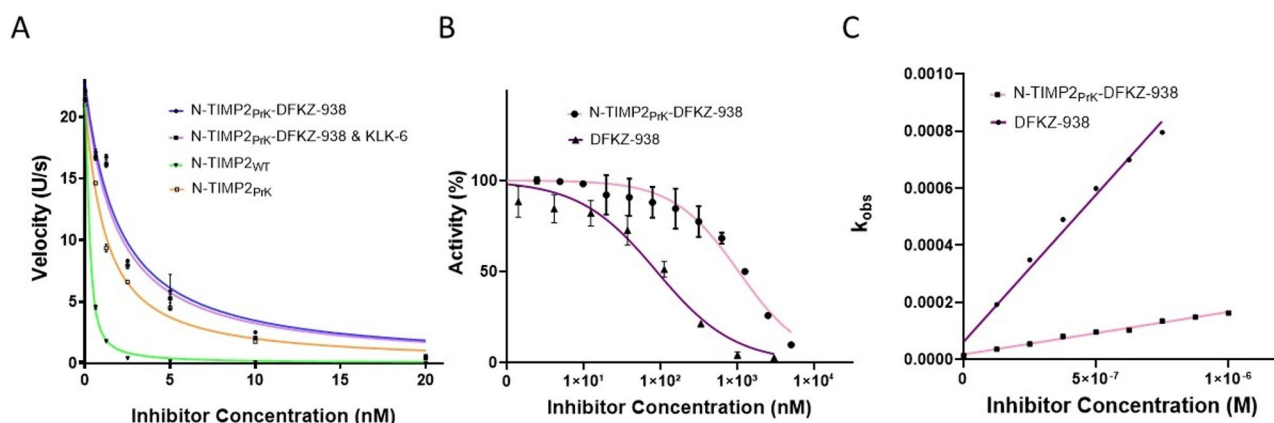


Fig. 1. Inhibition of the catalytic activities of MMP-9 and KLK6. **(A)** Tight-binding curves for inhibition of MMP-9_{CAT} catalytic activity by N-TIMP2_{WT}, N-TIMP2_{PrK}, and N-TIMP2_{PrK}-DKFZ-938 in the absence and presence of KLK6. **(B)** IC_{50} titration curve of DKFZ-938 and N-TIMP2_{PrK}-DKFZ-938 interacting with KLK6. The X-axis is on a logarithmic scale. **(C)** Double-reciprocal plot of k_{obs} (observed rate of the inactivation) vs. inhibitor concentration to determine k_{inact} and K_i values. The k_{inact}/K_i ratio, reflecting the efficiency of enzyme inactivation, was calculated from the slope. Results are given as means $\pm$ SD ($n = 3$). Raw kinetic progress curves are presented in the Supplementary Information.

	N-TIMP2 _{WT}	N-TIMP2 _{PrK}	DKFZ-938	N-TIMP2 _{PrK} -DKFZ-938
Ki for MMP-9 _{CAT} (nM)	0.034 ± 0.002	0.396 ± 0.033	N.A.	0.679 ± 0.089 (in the absence of KLK6) 0.646 ± 0.079 (in the presence of KLK6)
IC ₅₀ for KLK6 (μM)	N.A.	N.A.	0.09 ± 0.02	1.0 ± 0.2
k _{inact} /K _i (M ⁻¹ s ⁻¹)	N.A.	N.A.	1030 ± 60	148 ± 18

Table 1. Inhibition of MMP-9_{CAT} and KLK6 by the Inhibitors, N-TIMP2_{PrK} and DKFZ-938, individually and as a conjugate. N.A. - not applicable.

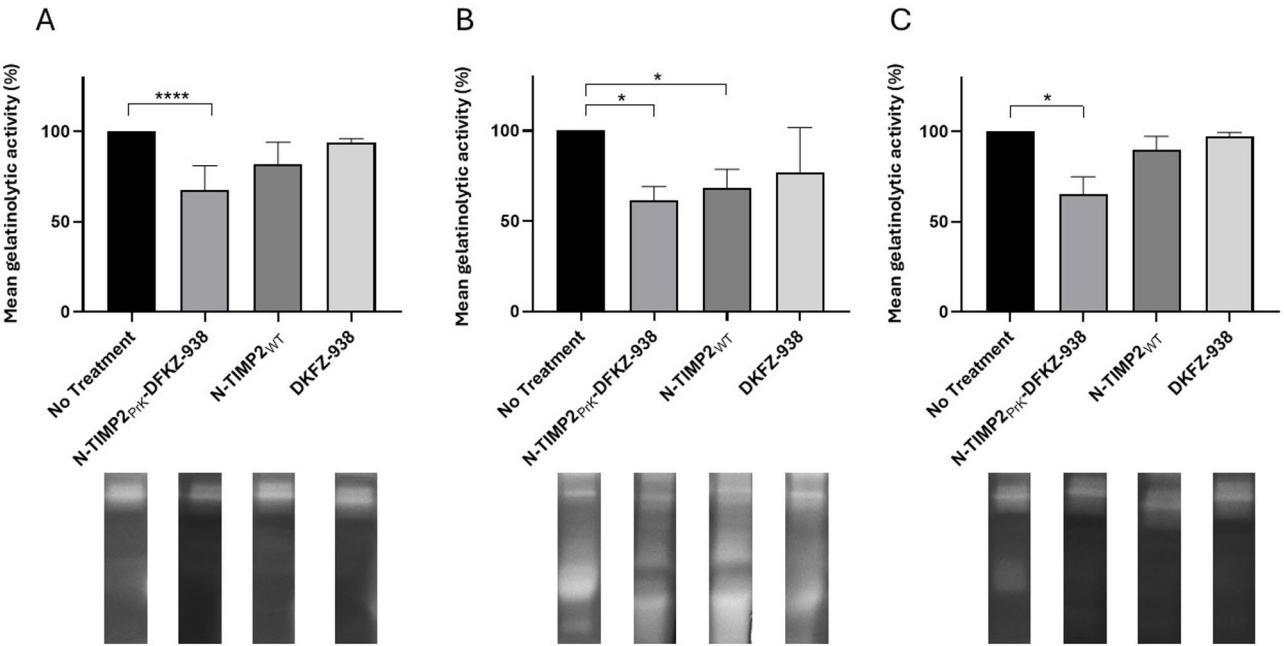


Fig. 2. Zymography analysis of cell-secreted MMP-9 and KLK6. Zymography analysis of MMP-9 and KLK6 gelatinolytic activity in PANC-1 (A), CAPAN-2 (B), and HCT-116 (C) cells following treatment with 500 nM N-TIMP2_{PrK}-DKFZ-938, N-TIMP2_{WT} or DKFZ-938. After electrophoresis, gels were incubated in developing buffer containing the above inhibitors for 48 h. Statistical significance was assessed using two-way ANOVA; **p* < 0.01, *****p* < 0.001. Results are given as means ± SEM (*n* = 3).

9_{CAT}^{61,62} Importantly, the presence of KLK6 does not alter the Ki value obtained for the inhibition of MMP-9_{CAT} by N-TIMP2_{PrK}-DKFZ-938, indicating that KLK6 does not interfere with the inhibitory activity of the conjugated protein (Fig. 1A; Table 1). As expected from structural considerations, a more pronounced reduction in inhibitory efficiency was obtained for KLK6 than for MMP-9_{CAT} upon conjugation of N-TIMP2_{PrK} to DKFZ-938. This stronger effect may be attributed to the steric hindrance imposed by the larger N-TIMP2_{PrK} protein vs. the DKFZ-938 small molecule, in keeping with the phenomenon previously reported for other protein–small molecule conjugates.^{63,64}

N-TIMP2_{PrK}-DKFZ-938 inhibits MMP-9 activity in cancer cell lines

The presence of cell-secreted KLK6 and MMP-9 was confirmed using Western blot analysis of serum-free medium collected from cultures of the pancreatic (CAPAN-2 and PANC-1) and colorectal (HCT-116) cell lines, whose invasiveness is known to depend on MMP-9 and KLK6 catalytic activity (Figure S4).^{65,66} In zymography on SDS-PAGE⁶⁷ of culture media taken from PANC-1, CAPAN-2 and HCT-116 cells, N-TIMP2_{PrK}-DKFZ-938 demonstrated superior inhibition (> 30%) of MMP-9 and KLK6 gelatinolytic activity vs. N-TIMP2_{PrK} and DKFZ-938 alone, thereby indicating the potential of N-TIMP2_{PrK}-DKFZ-938 to interfere with protease-mediated ECM degradation in these cancer models. Since similar MMP-9 inhibition was obtained for N-TIMP2_{PrK}-DKFZ-938 and N-TIMP2_{WT} and, as expected, no inhibition was observed for DKFZ-938 alone, it is suggested that conjugation of DKFZ-938 to N-TIMP2_{PrK} does not interfere with the interaction of the N-TIMP2_{PrK} part of the conjugate with MMP-9 (Fig. 2).

N-TIMP2_{PrK}-DKFZ-938 inhibits migration and invasion of cancer cells

To assess whether the bispecific inhibitor N-TIMP2_{PrK}-DKFZ-938 effectively inhibits cancer cell migration and invasion, we performed a series of transwell migration assays with PANC-1, CAPAN-2, and HCT-116 cells, all of which co-express MMP-9 and KLK6 (Figure S4).⁶⁸ Dose-dependent inhibition of cell migration was

observed in all three cell types following treatment with increasing concentrations of N-TIMP2_{PrK}-DKFZ-938 (Fig. 3A–C). The observed inhibition of migration was statistically significant (vs. untreated cells), supporting the functional impact of dual protease targeting in the ‘migratory context’. Subsequently, we compared the bispecific inhibitor to its monospecific components and their combination in admixture. Notably, for all the tested cells, N-TIMP2_{PrK}-DKFZ-938 demonstrated superior inhibition of migration in comparison with both its monospecific counterparts (Fig. 3D–F), although its superior inhibition in comparison with the admixture was evident only for PANC-1 (D) and HCT-116 (F). This latter finding highlights the benefit of simultaneous inhibition of the two targets with a single bispecific molecule, which is likely driven by binding avidity (i.e., the combined strength of two simultaneous interactions), despite the fact that DKFZ-938 is cleaved and splits away from N-TIMP2 following KLK6 inhibition.²⁷

To confirm the results of the migration experiment, we performed a scratch assay.⁶⁹ The dose-dependent inhibitory effect of N-TIMP2_{PrK}-DKFZ-938 on the migration of PANC-1, CAPAN-2 and HCT-116 cells observed in that assay (Fig. 4A–C) was indeed consistent with the transwell assay results. Similarly, N-TIMP2_{PrK}-DKFZ-938 showed superior inhibition of migration compared to the monospecific inhibitors and their combination (Fig. 4D–F), further supporting the functional advantage of simultaneous dual-target inhibition. The observed variability in response between the three cell lines in the scratch assay may be attributed to differences in the endogenous expression and activity of MMP-9 and KLK6 as well as intrinsic cellular characteristics, such as proliferation rate and adhesion properties, which together could influence the extent of inhibition observed at the same inhibitor concentration.⁷⁰ In addition, the apparent differences in the percentage of inhibition of migration with 1000 nM N-TIMP2_{PrK}-DKFZ-938 in CAPAN-2 and HCT-116 cells (Fig. 4B vs. E and Fig. 4C vs. F) likely reflect inter-experimental variability. For all the tested cell lines and treatments, migration was not completely abolished, suggesting the involvement of additional proteases beyond MMP-9 and KLK6 in driving cell motility. Such redundancy is well documented, as cancer cells often exploit multiple proteolytic pathways, including other MMPs and kallikreins, to facilitate migration and invasion.⁷¹ For instance, MMP-9 can also promote the epithelial–mesenchymal transition via PAR1 activation, illustrating the diverse downstream effects through which proteases contribute to motility.⁷²

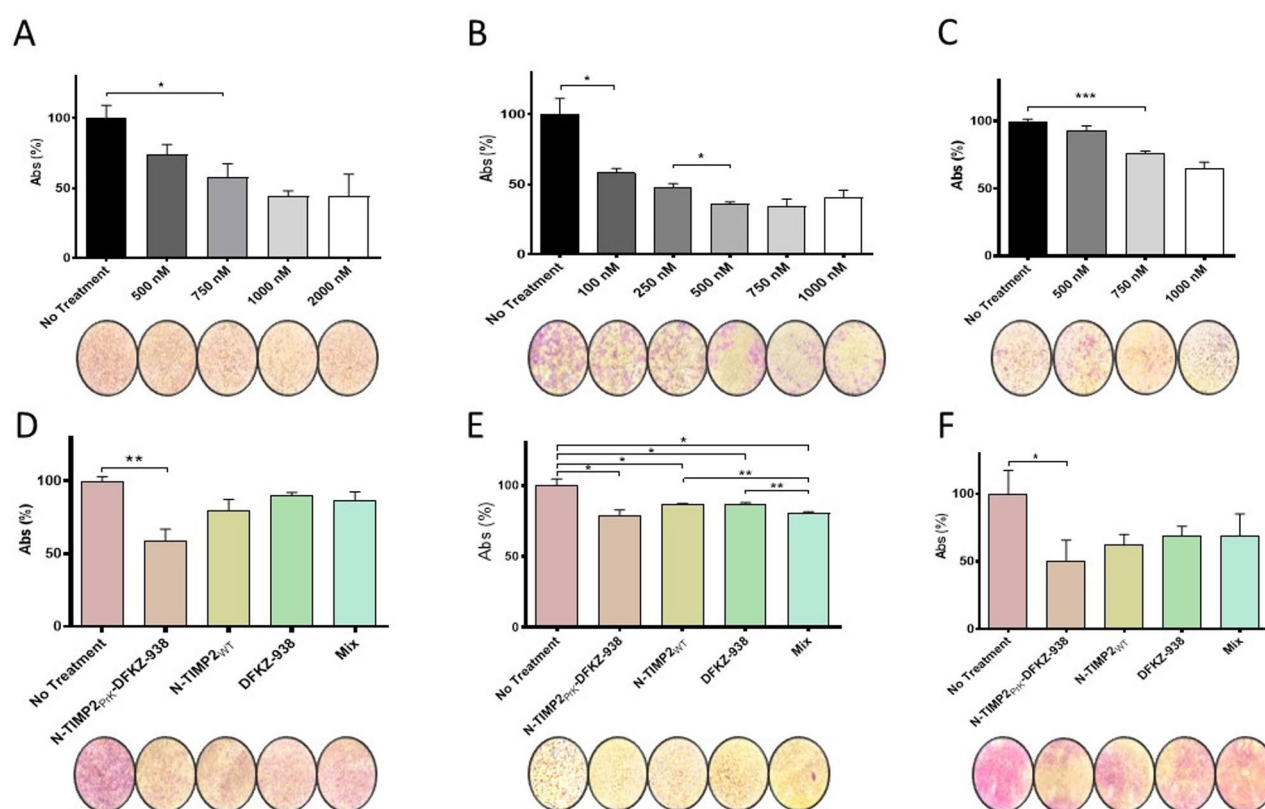


Fig. 3. Inhibition of cell migration by N-TIMP2_{PrK}-DKFZ-938 in cancer cell lines, as determined in a transwell assay. (A–C) Concentration-dependent inhibition of migration in PANC-1 (A), CAPAN-2 (B), and HCT-116 (C) cells by N-TIMP2_{PrK}-DKFZ-938. Statistical significance was determined by Student's t-test, comparing each concentration to the preceding lower concentration; * $p < 0.01$, *** $p < 0.001$. (D–F) Inhibition of migration in PANC-1 (D), CAPAN-2 (E), and HCT-116 (F) cells by 1000 nM N-TIMP2_{PrK}-DKFZ-938, N-TIMP2_{WT}, DKFZ-938, or a mixture of N-TIMP2_{WT} and DKFZ-938. Statistical significance was determined by Student's t-test, comparing treatments to one another and to the untreated control; * $p < 0.01$, ** $p < 0.005$. Representative brightfield images of the stained membranes are shown as circles below each graph. Results are given as means $\pm$ SEM ($n = 3$).

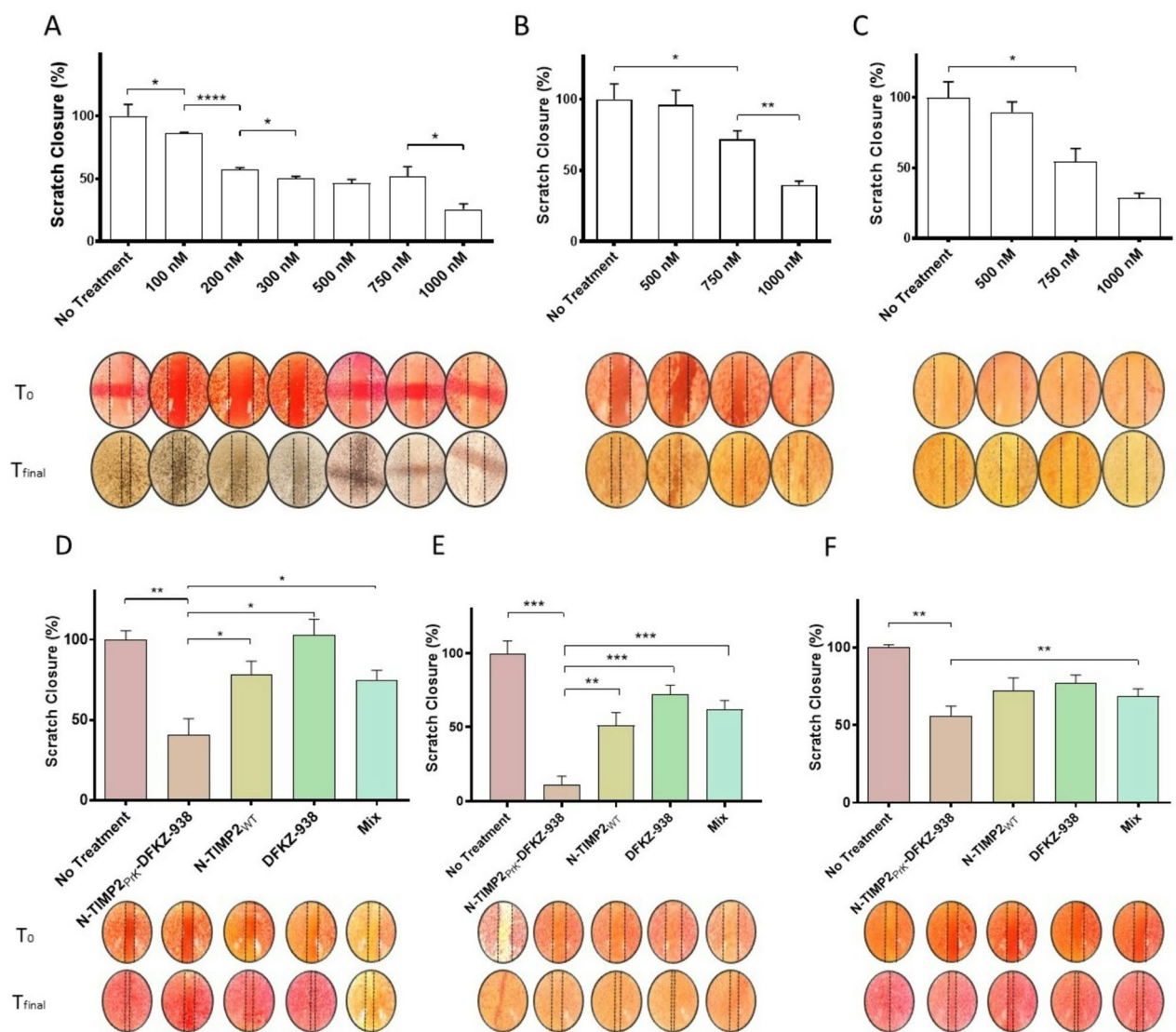


Fig. 4. Inhibition of cell migration in cancer cell lines, as determined by a scratch assay. Scratch closure was evaluated in PANC-1 (A, D), CAPAN-2 (B, E), and HCT-116 (C, F) cells following treatment with increasing concentrations of N-TIMP2_{PrK}-DKFZ-938 (A–C), as well as with 1000 nM N-TIMP2_{WT}, DKFZ-938, or a mixture of N-TIMP2_{WT} and DKFZ-938 (designated “Mix”) (D–F). Representative brightfield images of the wells are shown as circles below each graph. Results are given as means ± SEM ($n = 3$). Statistical significance was determined by Student’s t-test, comparing treatments to one another and to the untreated control; * $p < 0.01$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0005$.

The findings obtained from both the scratch and transwell migration assays are in agreement with other studies that highlight the critical role of MMP-9 in promoting cell motility, and with those that show dose-dependent inhibition of cell migration following specific targeting of MMP-9. For example, peptides mimicking the outermost β -strands of blades I and IV of the MMP-9 hemopexin domain were shown to inhibit MMP-9-mediated migration of transfected COS-1 cells in a transwell assay,⁷³ and small molecules targeting MMP-9^{CAT} were shown to reduce migration of the cells, although inhibition was only partial (50–60%) and was obtained at high inhibitor concentrations (μ M range).⁷⁴ In contrast, in our transwell assays, N-TIMP2_{PrK}-DKFZ-938 conferred significant inhibition of cell migration at concentrations as low as the low micromolar range in all three tested cancer cell lines, thus demonstrating the markedly higher potency of the conjugate. N-TIMP2_{PrK}-DKFZ-938 also exhibited superior inhibition of cell migration in comparison with the inhibition (~40–50%) obtained by knockdown of MMP-9 (using shRNA) in oral squamous cell carcinoma cells (SCC15 and CAL27).⁷⁵ Similarly to the inhibition of MMP-9, inhibition of KLK6 has also been shown to significantly reduce cancer cell migration. For example, in a transwell ovarian cancer cell migration assay, KLK6 inhibition resulted in a dose-dependent reduction of migration, substantiating the role of KLK6 in facilitating cell motility.⁷⁶ Moreover, silencing of KLK6 in gastric cancer cells decreased migration in both transwell and scratch assays, confirming its contribution to both migration and invasion of cancer cells.⁷⁷

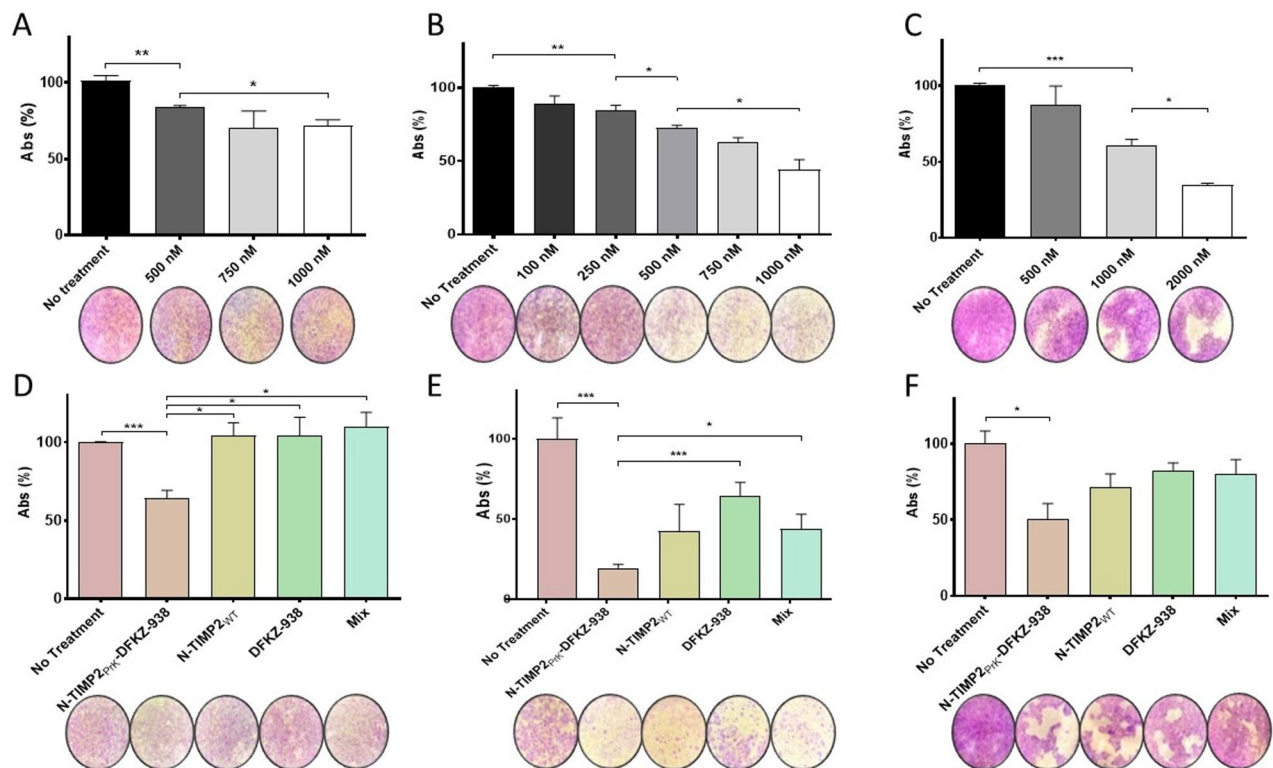


Fig. 5. Inhibition of cancer cell invasion. (A–C) Dose-dependent inhibition of invasion by N-TIMP2_{PrK}-DKFZ-938 in PANC-1 (A), CAPAN-2 (B), and HCT-116 (C) cells. (D–F) Inhibition of invasion by 1000 nM N-TIMP2_{PrK}-DKFZ-938, N-TIMP2_{WT}-DKFZ-938, and a mixture of N-TIMP2_{WT} and DKFZ-938 (designated “Mix”) in PANC-1 (D), CAPAN-2 (E), and HCT-116 (F) cells. Representative brightfield images of the stained membranes are shown as circles below each graph. Results are presented as means ± SEM ($n=3$). Statistical significance was determined by Student’s t-test, comparing treatments to one another and to the untreated control; * $p < 0.01$, ** $p < 0.005$, *** $p < 0.001$.

To investigate the effect of our bispecific inhibitor on the invasion of PANC-1, CAPAN-2 and HCT-116 cells, we conducted a transwell invasion assay (with Matrigel-coated inserts) with N-TIMP2_{PrK}-DKFZ-938 and its monospecific counterparts, alone and in admixture. Dose-dependent inhibition of invasion was obtained for N-TIMP2_{PrK}-DKFZ-938 (Fig. 5A–C), and for 1000 nM of the three tested compounds inhibition was significant only for TIMP2_{PrK}-DKFZ-938 (vs. the monospecific controls and their mixture) in all the tested cells (Fig. 5D–F). Importantly, N-TIMP2_{PrK}-DKFZ-938 did not affect cell viability under the experimental conditions used, indicating that the observed inhibition of migration and invasion is not due to cytotoxic effects (Figure S5).

Previous studies have shown that selective inhibition of either MMP-9 or KLK6 can attenuate cancer cell invasion, albeit typically with relatively high inhibitor concentrations.^{74,76} More recently, engineered N-TIMP2 variants with high specificity for MMP-9 were reported to achieve strong inhibition of invasion without inducing cytotoxicity.⁷⁸ In the present study, the bispecific inhibitor N-TIMP2_{PrK}-DKFZ-938 induced strong and consistent inhibition of invasion at low nanomolar concentrations across all tested cell lines, whereas previously described monospecific inhibitors generally required micromolar concentrations to achieve comparable effects. This finding – together with the findings of the transwell and scratch assays showing the superior and consistent inhibition by N-TIMP2_{PrK}-DKFZ-938 of cell migration – underscores the markedly enhanced potency and functional advantage achieved by simultaneous targeting of both MMP-9 and KLK6 within a single molecular framework and further supports the potential advantage of dual-targeting strategies for suppressing metastatic behavior in cancer.

The monospecific inhibitors N-TIMP2_{WT} and DKFZ-938 alone exhibited only moderate inhibition of cancer cell migration and invasion, which may be a result of cellular compensatory mechanism(s). For example, blocking a single extracellular protease can trigger compensatory pathways, as KLK6 participates in complex proteolytic networks, including the activation of proMMP-2 and other proteases, such as ADAMTS-19,⁷⁹ and its inhibition can promote E-cadherin shedding, leading to additional ECM remodeling through other proteases.⁸⁰ Notably, KLK6 inhibition has also been reported to increase the secretion of alternative serine and lysosomal proteases.⁸¹ In line with these compensatory effects, a mixture of the two monospecific inhibitors did not enhance inhibition relative to each inhibitor alone, in similar concentrations, likely because the separate molecules diffuse independently into the extracellular environment and cannot efficiently target both MMP-9 and KLK6 at the same time. Despite the cleavage of the conjugate molecule into the two monospecific inhibitors, DKFZ-938 and N-TIMP2_{PrK}, upon binding to (and hence inhibiting) KLK6 (Figure S6), the bispecific inhibitor N-TIMP2_{PrK}-

DKFZ-938 simultaneously engages both proteases, with enhanced affinity due to avidity effects, limiting compensatory responses and achieving stronger suppression of migration and invasion. This underscores the mechanistic and functional advantage of dual targeting with a single integrated molecule.

In summary, our bispecific inhibitor N-TIMP2_{PrK}-DKFZ-938 consistently demonstrated significantly greater efficacy in inhibiting both migration and invasion compared to its monospecific components and their combination. Importantly, despite the pronounced inhibition of metastatic traits, cell viability remained unaffected across all tested concentrations of N-TIMP2_{PrK}-DKFZ-938, reinforcing the notion that the observed effects are indeed due to targeted inhibition of KLK6 and MMP-9 rather than to their general cytotoxicity. While the overall efficacy of N-TIMP2_{PrK}-DKFZ-938 is comparable to other highly potent monospecific inhibitors reported in the literature,^{74,76,78} its dual-targeting design provides a mechanistic advantage by simultaneously engaging both proteases, thereby limiting compensatory protease activity and enabling potent suppression of metastatic behaviors.

Methods

Production and purification of N-TIMP2_{WT} and N-TIMP2_{PrK}

The pEVOL vector (a kind gift from Prof. Lital Alfonta, BGU, Beer-Sheva, Israel), containing *Methanosarcina mazei* PylRS/tRNA_{CUA}^{Pyl} genes for the incorporation of PrK, was cotransformed into *E. coli* strain WK6 with the pMECS plasmid (a kind gift from Dr. Serge Muyldermans, Vrije University of Brussels, Brussels, Belgium) bearing N-TIMP2-S31TAG. *E. coli* strain WK6 transformed with the pMECS plasmid containing the wild-type N-TIMP2 gene served as the control. The bacteria were grown, with stirring at 200 rpm, at 37 °C in TB medium (17 mM KH₂PO₄, 94 mM K₂HPO₄, 12 g/L peptone, 24 g/L yeast extract, 0.4% glycerol), containing 100 µg/mL ampicillin for N-TIMP2 or 100 µg/mL ampicillin, 50 µg/mL chloramphenicol, and 1 mM PrK (SynChem, Inc, Elk Grove Village, USA) for N-TIMP2-S31TAG. At an OD₆₀₀ of 0.4, 0.2% arabinose (Mercury, Rosh Ha'ayin, Israel) was added to N-TIMP2-S31TAG (for PylRS induction), and at an OD₆₀₀ of 0.6–0.9, the expression of both proteins (N-TIMP2_{WT} and N-TIMP2_{PrK}) was induced by addition to the medium of 1 mM IPTG (Sigma-Aldrich, Israel) and overnight incubation at 28 °C. Periplasmic extracts, containing the soluble proteins, N-TIMP2_{WT} and N-TIMP2_{PrK}, were obtained by osmotic shock using 9 mL of TES buffer (500 mM sucrose, 200 mM Tris-HCl, 0.5 mM EDTA, pH 8) per cell pellet [generated from 500 mL of culture (with a final OD₆₀₀ >20)], for 3 h at 4 °C and 200 rpm, followed by overnight incubation with the addition of 18 mL of TES buffer (diluted 1:4 in doubly distilled water). The proteins were purified using affinity chromatography on Ni-NTA gravitational beads (Invitrogen, CA, USA) and eluted with 0.5 M imidazole (Sigma-Aldrich, Israel) in phosphate-buffered saline (PBS). The eluted fraction was dialyzed against PBS, and the size and purity of the proteins were evaluated using SDS-PAGE gel electrophoresis and mass spectrometry (MALDI-TOF Reflex-IV, Ilse Katz Institute for Nanoscale Science and Technology, BGU, Israel). Protein concentrations were determined by UV–vis absorbance at 280 nm, using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA), with an extinction coefficient (ε₂₈₀) of 13,500 M⁻¹ cm⁻¹ for all N-TIMP2 variants. To confirm the incorporation of PrK by monitoring the expression of N-TIMP2_{PrK}, a small-scale experiment was conducted overnight in 10 mL of TB medium, following the same growth, induction, and expression conditions described above, with the exception that PrK was omitted to assess PrK-dependent full-length protein expression. Protein expression was determined by western blotting, using a 1:2000 dilution of mouse anti-6×His primary antibody (Abcam, Cambridge, U.K.), followed by a 1:2000 dilution of anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) (Cell Signaling Tech, Rehovot, Israel) and detection by chemiluminescent reagents (EZ-ECL, Biological Industries, Israel).

Proof-of-concept for the click chemistry reaction

N-TIMP2_{PrK} was labeled using a Cu(I)-catalyzed azide–alkyne cycloaddition reaction, as previously described with some modifications.⁸² Briefly, N-TIMP2_{PrK} was added to a reaction solution containing 2.5 mM 3,3',3''-(4,4',4'' (nitrilotris(methylene))tris(1 H-1,2,3-triazole-4,1-diyl))tris(propan-1-ol) (THPTA; Sigma-Aldrich, Rehovot, Israel), 0.5 mM CuSO₄, and 0.05 mM Cy5.5-Az (Lumiprobe, MD, USA). Ascorbic acid was added to a final concentration of 5 mM, and the reaction mixture (50 µL) was incubated at room temperature for 1 h. SDS-PAGE analysis on an 18% polyacrylamide gel was then performed under reducing conditions. The labeled protein was visualized in-gel using a Typhoon FLA 9500 imager (GE Healthcare Life Sciences, Uppsala, Sweden), set in the Cy5 fluorescence mode (excitation: 649 nm; emission: 670 nm) for Cy5.5-Az. Subsequently, the mixture was washed with PBS using an Amicon-Ultra 15 with a 3-kDa cutoff (Merck, Darmstadt, Germany) to remove any excess unbound dye. Finally, the concentration of the conjugated protein was determined using a colorimetric BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Production of N-TIMP2_{PrK}-DKFZ-938

N-TIMP2_{PrK} was conjugated to DKFZ-938 by using a click chemistry reaction, as previously described with some modifications.⁸² For this reaction, CuSO₄ (0.5 mM) and THPTA (2.5 mM) were premixed for 20 min at room temperature. Thereafter, DKFZ-938 was added to a final concentration of 0.05 mM (at a 1:5 molar ratio of N-TIMP2_{PrK} to DKFZ-938), followed by addition of N-TIMP2_{PrK} (0.01 mM) and ascorbic acid (to a final concentration of 5 mM). The tube containing the reaction mixture was then sealed and gently rocked at 37 °C for 2 h. Subsequently, the mixture was washed four times with PBS using Amicon-Ultra 15 with a 3-kDa cutoff (Merck) to remove any residual DKFZ-938. The work-up was completed as described above. The concentration of N-TIMP2_{PrK}-DKFZ-938 was determined using a colorimetric BCA protein assay kit (Pierce Biotechnology), and the successful formation of the conjugated product was confirmed by SDS-PAGE and MALDI-TOF MS, as shown in Figure S3.

MMP-9 inhibition studies

Inhibition of MMP-9_{CAT} by N-TIMP2 proteins was determined as previously described.⁸³ The K_i for the catalytic activity of MMP-9_{CAT} (0.325 nM) against the fluorogenic substrate, Mca-Pro Leu-Gly-Leu-Dpa-Ala-Arg-NH₂-TFA (7.5 μM; Merck Millipore, CA, USA), was determined for N-TIMP2_{WT}, N-TIMP2_{PrK}, and N-TIMP2_{PrK}-DKFZ-938, all at 0–12.5 nM. K_i values were calculated according to Morrison's equation (Eq. 1), the classic competitive inhibition equation for tight binding, using Prism (GraphPad Software, Version 11.0.2, <https://www.graphpad.com>). Mean values of $K_i \pm$ standard error of the mean (SEM) were obtained from three independent experiments.

$$\frac{V_i}{V_0} = 1 - \frac{([E] + [I] + [K_i^{app}]) - \sqrt{([E] + [I] + [K_i^{app}])^2 - 4[E][I]}}{2[E]} \quad (1)$$

where V_i is the enzyme velocity in the presence of an inhibitor, V_0 is the enzyme velocity in the absence of an inhibitor, $[E]$ is the enzyme concentration, $[I]$ is the inhibitor concentration, $[S]$ is the substrate concentration, K_m is the Michaelis–Menten constant, and K_i^{app} is the apparent inhibition constant, which is given by Eq. 2:

$$K_i^{app} = K_i \left(1 + \frac{[S]}{K_m} \right) \quad (2)$$

where K_i is the inhibition constant.

KLK6 inhibition studies

KLK6 was purified at the Protein Expression and Purification Core Facility (EMBL Heidelberg, Germany), as previously described.^{27,84,85} KLK-6 enzymatic activity was monitored in vitro in terms of cleavage of the fluorogenic substrate, N-Boc-FSR-AMC (Bachem), with a monochromator CLARIOstar[®] plate reader (BMG LABTECH). In each well of a 384-well black plate, 100 μL of inhibitor at 0–5000 nM for N-TIMP2_{PrK}-DKFZ-938 and at 0–3000 nM for DKFZ-938 or the DMSO control (final assay concentration of 2%) was added with 50 μL of substrate (final assay concentration of 300 μM) in reaction buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.05% Tween-20, pH 7.5) at room temperature. Then, 50 μL of KLK-6 (final assay concentration of 10 nM) in reaction buffer was added to each well immediately before starting the kinetic measurement of the fluorescence intensity (excitation, 355–20 nm; emission 460–40 nm; every 71 s for 70 min). Dose–response curves and IC₅₀ values were generated from the slope of the measured signals [normalized vs. the DMSO control (100% activity)] by using Prism, GraphPad Software. For k_{inact}/K_i determinations, a similar protocol was used with final inhibitor concentrations of 0–1000 nM. Mean K_i values $\pm$ SEM were obtained from three independent experiments. Statistical analysis was performed using Student's t-test.

Cell lines

Cell cultures of human colon cancer cells HCT-116 and human pancreatic cancer cells PANC-1 and CAPAN-2 (American Type Culture Collection, Beit Haemek, Israel) were grown in the appropriate media at 37 °C and 5% CO₂. The culture media were supplemented with 10% tetracycline-free fetal bovine serum (FBS; Thermo Fisher Scientific), L-glutamine (2 mM), and penicillin (100 units/ml)/streptomycin (0.1 mg/mL) (Gibco, Modi'in, Israel). HCT-116 and PANC-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM), and CAPAN-2 cells were cultured in McCoy's 5 A medium.

Western blot analysis

Western blot analysis was performed to determine the expression of MMP-9 and KLK6 in cells. HCT-116, PANC-1 and CAPAN-2 cells were grown to 70–80% confluence in DMEM or McCoy's 5 A media, respectively, supplemented with FBS. The media were then replaced with serum-free media, and the cells were incubated for 48 h at 37 °C under 5% CO₂. The growth media, containing the secreted MMP-9 and KLK6, were collected and centrifuged for 10 min at 600 g. The supernatants were concentrated using Amicon-Ultra 15 with a 3-kDa cutoff (Merck). The concentrated samples were boiled in denaturing sample buffer and fractionated by electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, and then transferred to nitrocellulose membranes (Sigma-Aldrich, Israel). After blocking with 5% skim milk, membranes were incubated at 4 °C overnight with specific primary antibodies, namely, rabbit anti MMP-9 (Abcam, Cambridge, U.K.) and mouse anti KLK6 (ENCO, Israel), followed by a 1:2000 dilution of anti-rabbit and anti-mouse secondary antibody conjugated to HRP, respectively, and detection with chemiluminescent reagents (EZ-ECL).

Gelatin zymography assay

The gelatinolytic inhibitory activity of 500 nM N-TIMP2_{PrK}-DKFZ-938, N-TIMP2_{WT} and DKFZ-938 was determined in a gelatinase zymography assay, as previously described, with some modifications.⁸⁶ HCT-116 and CAPAN-2 cells were grown to 70–80% confluence in DMEM and McCoy's 5 A media, respectively, supplemented with 10% FBS. The media were then replaced with serum-free media, and the cells were incubated for 48 h at 37 °C under 5% CO₂. The growth media, containing the secreted MMP-9, were collected and centrifuged for 10 min at 600 g. The supernatants were concentrated using Amicon-Ultra 15 centrifugal concentrator with a 3-kDa cutoff (Merck), then loaded onto gelatin SDS-PAGE, washed for 1 h in 2.5% Triton X-100, and incubated overnight in a Novex[™] Zymogram Developing Buffer (Thermo Fisher Scientific) containing 200 nM of each treatment (i.e., N-TIMP2_{PrK}-DKFZ-938, N-TIMP2_{WT} and DKFZ-938) at 37 °C. Gels were stained with Simply Blue[™] SafeStain (Thermo Fisher Scientific) and captured with MiniBIS Pro (DNR Bio Imaging Systems, Neve

Yamin, Israel). The intensity of the white bands created by digestion of the gelatin by MMP-9 was quantified using ImageJ. A two-way ANOVA with multiple comparison to the untreated control was utilized for statistical analysis; Values are the means of three independent experiments.

Transwell migration assay

An in-vitro transwell assay was performed with ThinCert™ 24-well inserts (Greiner Bio-One, Kremsmünster, Austria) – in a set-up that mimics cellular migration from a nutrient-poor to a nutrient-rich environment – as previously described with minor modifications.⁸⁷ The lower compartment of the chamber was filled with 500 μ L of either DMEM (for HCT-116 and PANC-1), or McCoy's 5A medium (for CAPAN-2 cells), both supplemented with 10% FBS. The cells were seeded above the inserts (5×10^4 for HCT-116, 1×10^5 for PANC-1 and 4.5×10^5 for CAPAN-2) in 500 μ L of 0.5% FBS-containing media. The treatments (i.e., N-TIMP2_{PrK}-DKFZ-938, N-TIMP2_{WT}-DKFZ-938, and mixture of N-TIMP2_{WT} and DKFZ-938) were added to the ThinCert inserts. After 24 h of incubation at 37 °C under 5% CO₂, another dose of the relevant treatment was added to the ThinCert inserts, which were then incubated for an additional 24 h at 37 °C under 5% CO₂. Cells remaining above the insert membrane were removed with a cotton swab, and cells that had migrated through the insert were fixed in 25% methanol. After washing in 1×PBS, the cells were stained with 0.1% crystal violet (Sigma-Aldrich, Israel). The inserts were washed four times in doubly distilled water and air dried. Migrating cells were detected by light microscopy at 4× magnification. To quantify the migrating cells, 10% acetic acid was used to dissolve the cells, and absorbance was then measured at 595 nm using an Infinite M200 (TECAN, Männedorf, Switzerland). The experiment was performed in triplicate; statistical analysis was performed by Student's t-test;

Transwell invasion assay

An in-vitro transwell invasion assay was performed following the same procedure as for the transwell migration assay, with the sole modification that the inserts were coated with growth factor-reduced Matrigel (Corning, NY, USA), as follows⁸⁷: The inserts from a ThinCert 24-well cell culture plate (Greiner Bio-One, Kremsmünster, Austria) were coated with growth factor-reduced Matrigel diluted in serum-free medium at a 1:30 v/v ratio. After a 1-h incubation following the addition of Matrigel to the inserts, the cells were seeded above the inserts (5×10^4 for HCT-116, 2×10^5 for PANC-1 and 4×10^5 for CAPAN-2), and the assay proceeded as described above.

Scratch assay

The scratch assay for determining the inhibition of the migratory phenotype of HCT-116, PANC-1 and CAPAN-2 cell lines was performed as previously described.⁸⁸ Briefly, HCT-116, PANC-1, and CAPAN-2 cells (9×10^5 , 5×10^5 , and 8×10^5 cells per well, respectively) were cultured (in 10% FBS-supplemented DMEM or McCoy's 5A, 37°C, 5% CO₂) as confluent monolayers in a 24-well plate. After approximately 24 h, when the cells had reached 100% confluence, a scratch was created by removing a strip of cells across each well by using a P20 pipette tip. The scratched monolayers were then washed twice with medium supplemented with 10% FBS to remove non-adhering cells. After a third wash, the medium was replaced with 2% FBS-containing medium supplemented with the following treatments: increasing concentrations of N-TIMP2_{PrK}-DKFZ-938 (100–1000 nM) or a fixed concentration of 1000 nM for the various inhibitors (i.e., N-TIMP2_{PrK}-DKFZ-938, N-TIMP2_{WT} and DKFZ-938). The cells were captured using a light microscope at 4× magnification immediately after performing the scratch and every 24 h thereafter (up to 96 h post-scratch) until the 'wound' had fully closed. The experiment was performed in triplicate, and the images were analyzed using ImageJ software (Version 1.54t, <https://imagej.net/ij/>). Wound closure (%) was calculated as [(initial area of the wound - final area)/initial area]·100 and normalized to the natural wound healing activity of each cell line (without any treatment). Statistical analysis for the comparison of the inhibitor to the natural activity was performed by Student's t-test.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

I.B., Y.P. and N.P. designed the research; I.B., Y.P. and A.B. performed the research; I.B., Y.P. and N.P. analyzed the data; I.B., Y.P. and N.P. wrote the paper. All authors edited the manuscript and approved the final version.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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