Targeting Ovarian Tumor Cell Adhesion Mediated by Tissue Transglutaminase

May Khanna1, Bhadrani Chelladurai2, Aruna Gavini2, Liwei Li1,6, Minghai Shao2, David Courtney2, John J. Turchi1,2,5, Daniela Matei1,3,5, and Samy Meroueh1,4,5,6,7

Abstract

Tissue transglutaminase (TG2) is a transpeptidase involved in protein cross-linking through generation of ε-(γ-glutamyl)lysine isopeptide bonds. It also promotes cell adhesion through interaction with fibronectin and facilitates formation of fibronectin–integrin complexes. This interaction is involved in tumor cell adhesion to the matrix and in the process of tumor dissemination. Its inhibition by small molecules may therefore be useful in blocking metastasis. To that end, we screened more than 800,000 compounds following an in silico docking approach targeting two distinct cavities in the vicinity of the fibronectin-binding site on TG2. A total of 120 compounds were acquired and tested in cell culture–based assays for inhibition of ovarian tumor cell adhesion and proliferation. Seven compounds showed more than 50% inhibition of cell adhesion at a concentration of 25 μmol/L. A follow-up fluorescence polarization study revealed that one compound in particular (ITP-79) inhibited binding of a TG2 peptide to a 42-kDa fragment of fibronectin in a dose-dependent manner. This inhibition was confirmed in cancer cells by coimmunoprecipitation. A competition assay with surface plasmon resonance showed that ITP-79 modulated binding of TG2 to fibronectin. Direct binding of compounds that inhibited adhesion to TG2 were examined with differential scanning fluorimetry, which measures the effect of the compound on the melting temperature of the target. Two compounds, including ITP-79, reduced TG2 stabilization, mimicking the effects of GTP, a known negative allosteric regulator of TG2 enzymatic function. This suggests a potential allosteric mechanism for the compound in light of its distal target site. Mol Cancer Ther; 10(4); 626–36. ©2011 AACR.
the establishment of metastases (8), such inhibitors may prevent cancer dissemination.

The 3-dimensional structure of TG2 (9) provided a starting point to conduct a computational search targeting 2 sites on the protein. A total of 120 compounds emerged from a screened library of 820,000. A solid-phase adhesion assay was carried out for all 120 compounds and identified several candidates that effectively inhibited cancer cell adhesion to fibronectin. Follow-up biochemical studies revealed that one compound inhibited the TG2–FN interaction. Coimmunoprecipitation studies confirmed that this compound blocked the TG2–FN interaction in ovarian tumor cells. To establish direct binding to TG2, we used differential scanning fluorimetry (DSF) to probe the effect of inhibitors of adhesion on the melting temperature of TG2. Results were compared to GTP, a known allosteric regulator of TG2 function. To the best of our knowledge, this work is the first attempt to use small molecules to modulate the interactions of TG2 with the extracellular matrix. Such molecules could serve as a foundation for the development of therapeutic agents to block metastasis in difficult-to-treat ovarian and pancreatic cancers.

Materials and Methods

Structure-based computational screening

The TG2 crystal structure with resolution of 2.0 Å was obtained from the RCSB Protein Data Bank (PDB code: 2Q3Z). Solvent molecules in the crystal structure were removed. The protein was protonated with the Reduce (version 3.03) program (10) to optimize the hydrogen bonding and van der Waals interactions. The TG2 structure was further processed using AutoDockTools (version 1.5.0, ref. 11) to assign Gasteiger charges and was used in the docking process. Ligand conformational search space was explored using the Lamarckian genetic algorithm. Each compound was docked 10 times, and the binding pose with the lowest binding energy was saved. The binding pose of each compound was further rescored using AutoGrid4, with the spacing of 0.375 Å.

Materials

Recombinant human TG2 was purchased from Neomarkers. Primary antibodies against TG2 and fibronectin were from Neomarkers, secondary horseradish peroxidase-conjugated antibodies were from Santa Cruz Biotechnology Inc. Unless stated otherwise, all chemicals and reagents were purchased from Sigma.

Cell lines

Human SKOV3 and OV90 ovarian cancer cell lines were obtained from the American Type Culture Collection and cultured in growth media containing 1:1 MCDB 105 (Sigma) and M199 (Cellgro) supplemented with 10% FBS and 1% antibiotics. IGROV-1 cells were a gift from Prof. L. Malkas (Indiana University) and cultured in RPMI (Sigma). The cells were not authenticated during the past 6 months by the authors.

Solid-phase adhesion assays

Trypsinized cells were labeled with calcein AM (Molecular Probes) and seeded at a density of 4 × 10^4 cells per well into 96-well plates precoated with 5 μg/mL fibronectin (Sigma) or bovine serum albumin (1% w/v) in the presence of the selected inhibitors. For the screening procedure, inhibitors were used at 25 μmol/L. After 60 minutes of incubation, the number of adherent cells was measured in a fluorescence plate reader (Applied Biosystems) at an excitation wavelength of 485 nm. Experiments were done in quadruplicate and repeated twice.

Coimmunoprecipitation

To detect the interaction between TG2 and fibronectin, SKOV3 cells were plated on fibronectin-coated dishes, allowed to adhere for 2 hours, and then lysed in a buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L β-glycerolphosphate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na3VO4, 1% Triton X-100, 10% glycerol, 1 mmol/L DTT (dithiothreitol), and protease inhibitors. After 30 minutes of incubation on ice, cells were collected and centrifuged to pellet cellular debris. For the separation of the membrane fraction, SKOV3 cells plated on fibronectin-coated dishes were collected in a hypotonic lysis buffer containing 10 mmol/L KCl, 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl, pH 7.4, and 2 mmol/L phenylmethylsulfonylfluoride (PMSF). The cell lysate was centrifuged at 4,000 × g for 15 minutes to remove cell debris and nuclei. The supernatant was then centrifuged at 100,000 × g for 60 minutes to separate the membrane fraction. The final crude membrane pellet was resuspended in a buffer containing 0.25 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 2 mmol/L PMSF. Equal amounts of protein (500 μg of cytosolic protein or 100 μg of cellular membrane fractions) were incubated with TG2 monoclonal antibody or immunoglobulin G (IgG; Santa Cruz Biotechnology). Immune complexes were incubated with protein A/G slurry (Calbiochem), then washed, eluted, separated by SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes (Millipore). After blocking, membranes were probed with antibodies against TG2 (CUB 7402; Neomarkers) and fibronectin.
(Neomarkers). After incubation with horseradish peroxidase-conjugated secondary antibody, antigen–antibody complexes were visualized using enhanced chemiluminescence (Amersham Biosciences).

**Cell proliferation**

Proliferation of SKOV3 cells was quantified by MTT assay after cells were incubated for 48 hours with each of the 120 inhibitors diluted to 5 mmol/L or vehicle. Treatment with the compounds started 24 hours after plating. The optical density was measured with an ELISA plate reader (SpectraMax 190) at 570 nm. All assays were done in triplicate. Data are presented as mean ± SEM.

**TG2 activity assay**

To measure TG2 enzymatic activity, the formation of hydroxamate from Nα-CBZ-glutaminyl-glycine and hydroxylamine was measured by using a colorimetric assay previously described (14, 15). In brief, 1 μg of purified TG2 (Sigma) was incubated with Nα-CBZ-glutaminyl-glycine and hydroxylamine in the presence of Ca2+ at 37°C and TGase activity was measured as the ability to generate hydroxamate. The colorimetric reaction was measured at 525 nm in an ELISA plate reader (SpectraMax 190). Selected inhibitors were added to the reaction at a 5 μmol/L concentration. All assays were done in duplicate. Data are presented as mean ± SEM.

**Differential scanning fluorimetry**

The melting temperature is measured by monitoring the increase of SYPRO orange that binds to hydrophobic regions of the protein (16, 17). Differential scanning fluorimetry (DSF) was conducted as previously outlined (16). A plate containing 25 μmol/L TG2, 4× SYPRO orange in 100 mmol/L HEPES, pH 7.4, and 150 mmol/L NaCl buffer was prepared. To each well, we added varying concentrations of compounds that were serially diluted, ensuring that the final concentration of DMSO was 2% across all samples. The thermal melting curves were obtained on a Light Cycler 480 by Roche. The melting temperature was obtained by normalizing the curves and obtaining the temperature at the midpoint of the transition curve.

**Fluorescence polarization**

The fluorescein-labeled human tissue TG2 hairpin peptide [FITC]RRRRRRRRDAVEEGDWTAVDQQD-CTLSLQLTTPANA (F-TG2R9) was obtained from Bio-synthesis, Inc. The 42-kDa gelatin-binding domain containing modules I6-II1-II2-I7-I8-I9 of human fibronectin (FN-42) was provided by Dr. Alexei Belkin of University of Maryland. Fluorescence anisotropy was conducted in a final volume of 100 μL containing 2 μmol/L F-TG2R9 and the indicated concentrations of FN-42. Excitation and emission wavelengths were 490 and 525 nm, respectively, with 5-nm slit widths. An auto cutoff filter at 515 nm was also used to prevent signal bleed over from a relatively narrow Stokes shift. Reactions were prepared in black 96-well plates (Costar) in 10 mmol/L Tris, pH 7.5.

Candidate inhibitor compounds (20 mmol/L) in DMSO were diluted to 2 mmol/L in 10 mmol/L Tris-Cl, pH 7.5, and screened at a final concentration of 50 μmol/L. The DMSO concentration was maintained at less than 1% for all assays.

**Surface plasmon resonance**

FN-42 was immobilized on a CM5 sensor chip (Biacore) at 20 μg/mL in 10 mmol/L sodium acetate, pH 4.5 (between 200 and 1,000 response units immobilized in duplicates), using the amine coupling kit according to manufacturer’s instructions (Biacore). Following immobilization, a few injections of running buffers (HBS-EP buffer; Biacore) were used to ensure that FN-42 was stable on the chip. Binding experiments were done in HBS-EP buffer (Biacore) at 10 μL/min flow, using 500 mmol/L TG2 and inhibitor concentrations ranging from 500 mmol/L to 50 μmol/L (injections of 40 μL volume were used with 240-second contact and 150-second dissociation times). Concentrations of DMSO were adjusted in the running buffer in addition to the samples injected such that the final concentration was maintained at 2% final DMSO concentration including the control sample with no inhibitor. Regeneration was done using a 5-μL injection of 50 mmol/L NaOH. We observed disruption of TG2 binding to fibronectin in the presence of inhibitors by monitoring the difference in surface plasmon resonance response. Compounds were also injected alone, and no refractive index was detected (data not shown).

**Statistical analysis**

For solid-phase adhesion, fluorescence polarization, and proliferation assays, we used the Student t test.

**Results**

**Computational search targeting TG2**

We focused our attention to the TG2 N-terminal 28-kDa fragment, especially the surface near amino acids 88–106, which was previously shown to be essential for fibronectin binding (18). Blind docking was first conducted to identify potential sites that can accommodate a small molecule around the fibronectin binding site. Unlike typical docking that is focused on a single binding cavity, blind docking considers the entire surface of the protein. The molecule is allowed to roll on the surface until the most energetically favored binding sites are identified. When carried out a large number of times, molecules will accumulate at sites that lead to the most favorable binding. The more favorable the binding pocket, the more molecules will bind to it. In the region around the N-terminal fibronectin-binding site, 2 sites that had the largest number of molecules bound to it were identified (Fig. 1A). It is of interest to note that sites do not fall directly on top of the surface occupied by amino acids 88–106. However, the expectation is that fibronectin and potentially integrins are large proteins that will occupy binding surface near the fibronectin-binding β-hairpin surface. Hence,
molecules that bind at these locations should impair the interaction of TG2 with fibronectin either directly or potentially through an allosteric mechanism. Large conformational changes in the TG2 structure have been reported for TG2 bound to a gluten peptide; this open conformation being implicated in abnormal activation of the enzyme associated with celiac disease (9).

About 820,000 compounds were docked to each of the binding pockets referred to as site 1 and site 2 (Fig. 1A). The inhibitors are shown in sticks colored in yellow (carbon), red (oxygen), and blue (nitrogen), FN, fibronectin. B–E, a close-up view of adhesion inhibitors bound to their target sites. Left, TG2 in ribbon representation, with the fibronectin (FN)-binding β-hairpin colored in red. Residues that interact with compounds are shown in capped-stick representation (white, blue, and red for carbon, nitrogen, and oxygen). Right, TG2 in surface representation with the fibronectin-binding β-hairpin surface colored in red.

Figure 1. Binding mode of TG2 inhibitors. A, ribbon representation of TG2 crystal structure (PDB code: 2Q3Z) with the inhibitors of cell adhesion docked at the sites (Site 1 and Site 2) that were targeted during the docking effort. The inhibitors are shown in sticks colored in yellow (carbon), red (oxygen), and blue (nitrogen), FN, fibronectin. B–E, a close-up view of adhesion inhibitors bound to their target sites. Left, TG2 in ribbon representation, with the fibronectin (FN)-binding β-hairpin colored in red. Residues that interact with compounds are shown in capped-stick representation (white, blue, and red for carbon, nitrogen, and oxygen). Right, TG2 in surface representation with the fibronectin-binding β-hairpin surface colored in red.

Compounds block tumor cell adhesion

To select compounds that inhibit cell adhesion to fibronectin, we used SKOV3 ovarian cancer cells that express endogenously TG2. We had previously shown using this cell line that TG2 knockdown by stable transfection of an antisense construct inhibited adhesion to fibronectin (4). In the first ranking of small molecules, we used the solid-phase adhesion assay in the presence of the selected compounds or vehicle (control). The compounds were diluted to a concentration of 25 μmol/L and were classified into 3 categories on the basis of the percentage inhibition of cellular adhesion: (i) noninhibiting (<25% inhibition of cell adhesion); (ii) moderately inhibitory (25%–50% inhibition); and (iii) potent inhibitors (>50% inhibition (Fig. 2A). The chemical structure of 8 compounds that showed equal or greater than 50% inhibition is shown in Fig. 2B. Interestingly, the compounds fall into 4 classes on the basis of their chemical structure.

An fluorescence polarization assay reveals TG2–FN inhibition

Effects of compounds on the TG2–FN interaction were measured by fluorescence polarization. The fluorescence polarization assay was developed in a high-throughput format for optimal screening. Initial experiments were done to optimize the sensitivity and reliability of the assay. TG2–FN peptide concentration of 2 μmol/L was selected from initial titration experiment (data not shown). Under these conditions, titration of FN-42 to a
final concentration of 25 μmol/L revealed a hyperbolic binding curve indicative of a single reversible binding event (Fig. 3A). From these data, we selected a concentration of 10 μmol/L for inhibitor studies. Candidate inhibitors were analyzed at a fixed concentration of 50 μmol/L in triplicate, and the results obtained showed that one compound (ITP-79) was capable of blocking the TG2–FN interaction (Fig. 3B). To confirm the inhibitory activity of ITP-79, we varied the concentration of the compound from 1 to 100 μmol/L and the results obtained showed a concentration-dependent decrease in depolarization, consistent with ITP-79 inhibiting the TG2–FN interaction (Fig. 3C).

**ITP-79 inhibits the TG2–FN interaction in ovarian cancer cells**

As compound ITP-79 blocked cellular adhesion in the preliminary screen and also inhibited the TG2–FN interaction in the fluorescence polarization assay, it was characterized in further detail. A dose–response curve showed that inhibition of cellular adhesion by compound ITP-79 was dose dependent (Fig. 4A and B) in 2 ovarian cancer cell lines that express TG2 endogenously (SKOV3 and IGROV1). In contrast, compound ITP-79 did not inhibit cellular adhesion to fibronectin in OV90 ovarian cancer cells, which do not express TG2 (Fig. 4C), supporting the concept that ITP-79 acts in a TG2-specific manner.

To test whether ITP-79 inhibited the interaction between TG2 and fibronectin in the cellular environment, we used communoprecipitation of whole cell and membrane proteins. For this, SKOV3 cells plated on fibronectin were incubated with control, with an antibody against the NH2-terminus of the enzyme 4G3 (positive control; ref. 19), or with ITP-79 for 2 hours before lysis. Immunoprecipitation with TG2 antibody, followed by Western blotting for fibronectin, showed that TG2 and fibronectin formed a complex (Fig. 4D), which was disrupted by the 4G3 antibody (Fig. 4E) or by

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**Figure 2.** Representative results of the screening using solid-phase adhesion assay to fibronectin. A, the assay measured adhesion of SKOV3 cells to fibronectin in the presence or absence of compounds diluted to a 25 μmol/L concentration. Columns represent average percentage of cells adhering to fibronectin relative to vehicle (control) ± SEM. Results from use of selected compounds are shown and classified according to the adhesion inhibiting properties. B, chemical structures of compounds that showed approximately 50% inhibition of tumor cell adhesion at 25 μmol/L concentration.
ITP-79 (Fig. 4F, whole cell lysates, and Fig. 4G, membrane fractions). These data show that ITP-79 inhibits the binding between TG2 and fibronectin at the level of the cellular membrane. However, incubation of SKOV3 cells with compounds inactive in the cellular adhesion screen (e.g., ITP-50 and ITP-74) did not disrupt the communoprecipitation of TG2 and fibronectin (Fig. 4H).

Compounds following different binding mechanisms to TG2

As shown in Fig. 2, we found that in addition to ITP-79, a total of 7 compounds inhibited tumor cell adhesion at a level of 50% or more. Inspection of their chemical structure reveals that they fall into distinct chemical classes. The first class includes ITP-16, 81, and 82. They all share a benzylpiperidine core moiety. However, it is worth mentioning that other compounds that possessed the same core, such as ITP-60, did not show any effect in inhibiting tumor cell adhesion, suggesting that the functional groups around the core structure play an important role. The other class includes ITP-18, 94, and 113, which are distinguished by their triazine core. ITP-18 and ITP-94 contain a morpholino-substituted triazine core. The triazine core in ITP-113 is instead substituted by a \( p \)-fluorophenylamino group; this being the only compound of this class that exhibited cell toxicity. Compound ITP-71 was within a class of its own. It is structurally distinct from the other compounds. It distinguishes itself by its 2-fold symmetry and a large number of rotatable bonds. Finally, ITP-79 was within a class of its own as shown in Fig. 2.

To determine whether compounds that inhibited tumor cell adhesion did so through a TG2-dependent mechanism, we used DSF. This technique monitors the fluorescence of a dye (SYPRO orange) that binds to hydrophobic regions of the protein and is a direct measurement of the extent of exposed hydrophobic surfaces of the protein. As the protein unfolds, the fluorescence of the dye increases as more hydrophobic regions are exposed. We tested 5 compounds, one from each chemical class, at a concentration of 50 \( \mu \)mol/L. We also tested the effects of GTP, a well-known allosteric regulator of TG2 (20), on the melting temperature of TG2. As shown in Fig. 5A, GTP destabilized TG2 significantly by nearly 5 K. Two other compounds showed similar effects, namely, ITP-79 and ITP-94. ITP-79 was significantly more active than ITP-94 or GTP, shifting the melting temperature of TG2 lower by nearly 7 K, nearly 2 degrees lower than GTP. ITP-94, on the other hand, showed only about a 1-K destabilization of TG2. The remaining 3 compounds, namely, ITP-16, ITP-71 and ITP-81, all led to stabilization of the TG2 receptor by 1, 2, and 2 K, respectively. It was interesting to note that the effect on TG2 observed from DSF corresponded nearly exactly to the binding site of the compounds. All compounds that emerged from docking to site 1 (ITP-16, 71 and 81) stabilized TG2, whereas compounds that emerged from site 2 (ITP-79 and 94) destabilized TG2.
Compound ITP-79 inhibits the TG2-FN interaction

We used surface plasmon resonance to study the effect of this compound on the TG2–FN protein–protein interaction. This technique is widely used to characterize the binding kinetics and thermodynamics of proteins (21).

One of the binding partners (ligand) is covalently attached to the biosensor chip on a dextran surface, whereas the other (analyte) is injected and passed over the ligand with a flow rate specified by the user. In the event of binding, an increase in the sensorgram signal is observed.
observed (16). In this work, we immobilized FN-42 on the CM5 chip dextran surface and injected TG2 along with increasing concentration of ITP-79. As shown in Fig. 5B, there is strong binding between TG2 and immobilized fibronectin with a distinct association and dissociation phase. When TG2 was injected in the presence of increasing concentration of compound, there was a systematic reduction in the maximum response of the sensorgram, suggesting that there was less binding of TG2 to fibronectin through competitive binding (Fig. 5C). These results provide further evidence that compound ITP-79 modulates the TG2–FN interaction.

**Compounds inhibit cell proliferation without affecting TG2 enzyme activity**

Compounds were also characterized on the basis of antiproliferative properties by using MTT assay after 48-hour incubation in the presence of compounds or vehicle. The compounds were diluted to a concentration of 5 μmol/L and were classified on the basis of the percentage inhibition of cellular proliferation into 3 categories: (i) noninhibiting (<25% inhibition of cell proliferation); (ii) moderately inhibitory (25%–50% inhibition); and (iii) potent inhibitors (>50% inhibition; see Fig. 6A). We observed minimal overlap between compounds with antiadhesion properties and those with antiproliferative properties. The top 15 compounds identified as potent inhibitors of cellular adhesion to fibronectin were characterized further for enzyme inhibition. As the catalytic domain of TG2 is distinct from the fibronectin-binding region, we did not anticipate that compounds inhibiting the TG2–FN interaction would inhibit TGase activity. Figure 6B shows percentage inhibition of TGase by selected compounds. Most compounds did not inhibit TGase, with several compounds exerting modest inhibition.

**Discussion**

The complexes formed by β-integrins with fibronectin anchor cells into the matrix and are enhanced by TG2 (1), a protein whose enzymatic function is controlled by GTP through an allosteric mechanism (22). The 3-dimensional structure of TG2 has provided valuable insight into its function (9, 23, 24). The arrangement of amino acids within the active site has elucidated the catalytic machinery by which the enzyme does transamidation. The distal nature of the GTP-binding site (23) was intriguing and alluded to an allosteric mechanism by which the nucleotide can turn the enzymatic activity on or off (20). In addition to the catalytic core of the enzyme, other domains at the N- and C-termini of the protein have distinct physiologic functions (Fig. 1). The N-terminus contains the binding site for fibronectin, as mutations along a β-hairpin peptide significantly impaired binding of fibronectin (2). It is reasonable to assume that the binding site of integrins is around the same region, as integrins and fibronectin come in direct contact. In this work, we sought small molecules that block TG2-modulated cell adhesion by concentrating our effort at the N-terminus of the protein around the β-hairpin loop (amino acids 88–106) that is well known to be the binding site of fibronectin (2). A search for binding cavities around these amino acids led to 2 sites, namely, site 1 and site 2, as shown in Fig. 1. Site 1 is located at the foot of the β-hairpin that binds...
to fibronectin, whereas site 2 is on the opposite side of the β-hairpin (Fig. 1). Our virtual screening effort targeted both sites. Our rationale was that site 1 is located so close to the fibronectin binding site that compounds binding to this site will either directly disrupt the interaction with fibronectin or at least affect integrin binding. For site 2, we conjectured the compounds might affect fibronectin binding through an allosteric mechanism.

The top candidates that emerged from the computational search were directly evaluated for inhibition of ovarian cancer cell adhesion to fibronectin. A total of 7 compounds were found to inhibit adhesion at a level of 50% or more, and another 11 compounds inhibited adhesion by 25 to 50%. To test whether these compounds affected binding of a TG2-derived peptide consisting of the fibronectin-binding β-hairpin, the fluorescence polarization assay was used. The results showed that one compound, namely, ITP-79, inhibited the TG2–FN complex in a dose-dependent manner (Fig. 3). To complement the fluorescence polarization studies, we carried out a competition assay by surface plasmon resonance including the full TG2 injected on immobilized fibronectin. The observed decrease on the maximum resonance reflected by the sensorgram data advocates that ITP-79 interferes with the ability of fibronectin to bind to TG2 (Fig. 5B).

Direct binding of compounds to TG2 was studied with DSF (16). Two of the 5 compounds tested (ITP-79 and ITP-94) shifted the melting temperature in a manner similar
with that of GTP, a known allosteric modulator of TG2 (23). Importantly, ITP-79 showed greater destabilization than GTP by nearly 2 K. Three other compounds enhanced the stability of TG2, suggesting a different binding mechanism. It was interesting that the effects of the compounds on the stability of TG2 correlated exactly with the location of their binding site. Compounds that bind to site 1 stabilized TG2, whereas compounds that bind to site 2 destabilized the protein. The similarity in the effects of GTP and ITP-79 on the protein stability supports an allosteric mechanism of regulation for this compound. As we have argued previously for other small molecules (25, 26), effects mediated through large distances are dynamic effects consisting of the shifting of the equilibrium toward conformational states that promote the event that is modulated by allosterly. That ITP-79 inhibits TG2 binding to fibronectin suggests that the compound promotes structures of TG2 that are unfavorable for binding to fibronectin. GTP may be acting in a similar manner, promoting conformational states unsuitable for catalysis, either through impairing binding of the substrate or by positioning the catalytic residues in unproductive conformations that impair the reaction pathway.

The compounds that stabilize TG2, on the other hand, may be directly interfering with fibronectin binding. As shown in Fig. 1, site 1 is directly juxtaposed to the surface occupied by the β-hairpin that has been previously shown to be critical in fibronectin binding. Given that the β-hairpin peptide is not fully exposed, it is highly likely that the fibronectin binding interface includes additional surface surrounding the peptide that may include site 1. Hence, compounds binding to site 1 may hinder fibronectin binding. Alternatively, it may be possible that site 1 is located at a site that is critical for integrin binding, given that integrin is known to bind directly to fibronectin (27–29). Using this mechanism, compounds would instead affect integrin binding to fibronectin and thus impair cell adhesion.

The effect of the compound on the TG2–FN interaction was also observed in the cellular milieu where ITP-79 disrupted ovarian cancer cell adhesion to fibronectin and directly inhibited the complex formed between the enzyme and fibronectin. This suggested that the compound is accessible to membrane-bound TG2 and effectively blocks the interaction with fibronectin and integrins. These results suggest that compounds from this class may exert inhibitory effects for several of the critical steps of cancer metastasis. In ovarian cancer in particular, integrin mediated cell–matrix and cell–cell adhesion is essential to peritoneal dissemination (30–32). Aside from interaction with the extracellular matrix, fibronectin–integrin interaction is important for the formation of multicellular aggregates that promote cell spreading in the peritoneal milieu (32–35). Our recent unpublished data support an important role for TG2 in the formation of ovarian cancer spheroids. It is conceivable that compounds that disrupt the interaction between TG2 and fibronectin also affect the formation of multicellular aggregates, the vehicle that enables peritoneal metastasis.

It was encouraging that compound ITP-79 showed little cell toxicity, suggesting that the inhibition of adhesion is likely not caused by off-targets effects. The presence of a carboxylate moiety on the molecule leads to a negative overall charge, which will likely pose a significant barrier toward cell penetration. This is supported from physicochemical properties computed by using the Qikprop computer program based on the chemical structure of ITP-79. The results indicate a Caco-2 permeability of 102, suggesting intermediate cellular permeability. The C log P value of ITP-79 was 3.5; well within the range of most small molecules approved for clinical use. Further optimization of the compound may increase its solubility.

It is of interest to note that ITP-79 violates none of Lipinski’s rule of five, suggesting that it can serve as a suitable foundation for the development of therapeutics to block metastasis. The results presented in this study point to TG2–FN interaction as a new targetable complex of potential significance to cancer therapy and identify ITP-79 as a novel inhibitor for this complex.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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