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Bifunctional inhibitors of urokinase and metalloproteinase-9 for cancer treatment - in silico evaluation.

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Matrix metalloproteinase-9 (MMP-9), and urokinase plasminogen activator (uPA) overexpression or/and increased activity are considered causative elements for cancer invasion and metastasis. These enzymes are degrading the extracellular matrix (ECM) providing space for cancer progression and cancer cell mobility. Process of angiogenesis, in which microvascular endothelial cells form blood vessels, requires local degradation of the underlying basal lamina to invade into the stroma proximal to cancer, and it strongly depends on the activity of MMP-9 and uPA as well. Malignant tumor invasion, cancer metastasis and angiogenesis have been documented as a fundamental factors in the morbidity and mortality among cancer patients, thus their inhibition can be exploited therapeutically. Numerous in vivo and in vitro studies have demonstrated that inhibition of proteolytic activity can reduce cancer invasion, tumor size and limit angiogenesis. Consequently human clinical studies were designed inhibiting urokinase or MMPs, but these target-specific inhibitors produce mixed results. One of the possible explanations could be that cancers are overexpressing more than one enzyme simultaneously; for instance urokinase and MMPs. Thus upregulated net proteolytic activity should be normalized rather than trying to inhibit single proteolytic enzyme. Therefore, starting from specific inhibitors we have created - in silico - several hybrid molecules that could inhibit both uPA and MMP-9. The best hybrid (UI1xAGB) had theoretical affinities of Ki = 1.61^{-9} mol for MMP-9 and Ki = 1.36^{-9} mol for uPA. In the future each individual hybrid would need to be successfully synthesized and checked in the in vitro and in vivo analyses.

metalloproteinase-9 | urokinase | inhibitor | molecular modeling

MMatrix metalloproteinase-9 (MMP-9), and urokinase plasminogen activator (uPA) overexpression or/and increased activity are considered causative elements for cancer invasion and metastasis. These enzymes are degrading the extracellular matrix (ECM) providing space for cancer progression and cancer cell mobility (1, 2). Process of angiogenesis, in which microvascular endothelial cells form blood vessels, depends on local degradation of the underlying basal lamina to invade into the stroma proximal to cancer, and it strongly depends on the activity of MMP-9 and uPA as well (3-5). Since malignant tumor invasion, metastasis and cancer angiogenesis have been documented as fundamental factors in the morbidity and mortality among cancer patients and their inhibition can be exploited therapeutically (5-7).

Urokinase is an activator of plasminogen that upon cleavage is converted into plasmin, which can degrade a broad spectrum of proteins. Urokinase is expressed in tissues, contrary to tissue plasminogen activator (tPA) which is present predominantly in the blood (8, 9). Therefore targeting uPA only will preserve plasmin activity necessary for dissolving fibrin blood clots and some other physiological processes (10-13).

There are few possible approaches to inhibit urokinase. One is use of plasminogen activator inhibitor-1 (PAI-1). PAI-1, also known as endothelial plasminogen activator inhibitor or serpin E1, is a protein that functions as the principal inhibitor of urokinase and tissue plasminogen activator. Plasminogen activator inhibitor-1 exists as an active, nonactive-latent, and cleaved form. It converts spontaneously from active form into latent form in physiological conditions with half life time equal to t1/2=2 hours. Only active PAI-1 is therapeutically relevant. Thus, to use PAI-1 in therapy half-life must be extended (14-16). Several mutants of PAI-1 were produced extending its activity up to more than 700 hours (17-19). The other approach is to use antibodies against active site of uPA to restrict plasmin driven proteolytic activity (20-23). Although small molecule binding into specificity pocket or proximity of catalytic triad might be the easiest to produce. Among the large number of small molecular inhibitors amiloride was found to be uPA specific (24-27). Moreover, optimization of amiloride's structure to potentiate inhibitory activity and loss of diuretic effects resulted in few novel anticancer compounds (25, 26, 28). Several clinical studies were conducted to evaluate inhibition of urokinase activity or expression on cancer cells (29-34). Also, limited number of studies were monitoring prevention of cancer related angiogenesis. These reports show potential benefit of anti urokinase therapy in cancer patients and emphasize needs for additional trials (29-34).

Pro-MMP-9 is activated by protease cascade involving plasmin and stromelysin 1 (MMP-3). Plasmin cleaves MMP-3 zymogen to form active MMP-3 that cleaves the propeptide from the 92-kDa pro-MMP-9, generating an 82-kDa enzymatically active enzyme (35). The active MMP-9 domain contains two zinc and three calcium ions necessary for its function. The catalytic zinc is coordinated by only three histidines while the other metal co-factors (zinc and the three calcium) have their coordination spheres fulfilled by the components of surrounding protein structure (36).

Inhibition of MMP-9 by small molecular chemicals lies on alteration of its activity or/and reduction of protein expression by acting on DNA or RNA (37, 38). Like in the case of urokinase, MMP-9 can be inhibited by antibodies. For example GS-5745 antibody inhibits MMP-9 by binding to pro-MMP-9 preventing activation of

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this metalloproteinase, or binding allosterically to active MMP-9 reducing its activity (39-42). Several clinical studies have been conducted in over 25 years (43-51). Overwhelming evidence from animal studies warranted these studies, but unfortunately were plagued with side-effects of orally-dosed MMP-9 inhibitors. Fingleton (52) stated that for chronic dosing, agents with MMP inhibitory efficacy are needed that show minimal toxicity at low concentration.

Given the well-known function for urokinase and MMP-9 in cancer cell invasion, metastasis and angiogenesis, a novel tactic to cancer therapy could be invented by testing inhibition of these proteases by one small molecular inhibitor. To inhibit both proteins at the same time, we have constructed in silico a novel hybrid compounds and evaluated their activity using Vina AutoDock program (53). This approach was used previously by constructing hybrid protein consisting of the tissue inhibitor of metalloproteinases (TIMP-1) linked to the ATF domain of u-PA (54, 55).

Materials and Methods

Chemicals. The following chemical structures were used in molecular simulations:

1. Amiloride, AMR (Urokinase inhibitor); 3,5-diamino-6-chloro-N-(diaminomethylidene)pyrazine-2-carboxamide.
2. Ab145190 (MMP-9 inhibitor); N-[(1,1'-Biphenyl)-4-ylsulfonyl]-D-phenylalanine.
3. UI1 (Urokinase inhibitor); N-[4-(aminomethyl)phenyl]-6-carbamimidoyl-4-(pyrimidin-2yl amino)naphthalene-2- carboxamide.
4. 7IN (Urokinase inhibitor); rac-(1Z,2R)-2-(benzylsulfonylamino)-3-hydroxy-N-[rac-(1S,2Z)-2-[(4-carbamimidoylphenyl) methylimino] -2-hydroxy-1-(hydroxymethyl)ethyl]propanimidic acid.
5. UI1xAGB (hybrid inhibitor); N-[4-[[2-[N-[4-[(1-adamantylcarb amoylamino)methyl] phenyl]carbamimidoyl] hydrazino] methyl] phenyl]-6-carbamimidoyl-4-(pyrimidin-2-ylamino)naphthalene -2-carboxamide.
6. UI1xAMR (hybrid inhibitor); N-[4-[[2-[6-amino-3-chloro-5-[(diaminoamino)carbamoyl]pyrazin-2-yl] hydrazino] methyl] phenyl]-6-carbamimidoyl-4-(pyrimidin-2-ylamino)naphthalene-2-carboxamide.
7. 7INxAMR (hybrid inhibitor); 3-amino-5-[4-[[2-[[2-[(4-carbamimidoyl phenyl) methylamino]-1-(hydroxymethyl)-2-oxo-ethyl] amino]-1-(hydroxymethyl)-2-oxo-ethyl]sulfamoylmethyl] anilino]-6-chloro-N-(diaminomethylene)pyrazine-2-carboxamide.
8. Hybrid3 (hybrid inhibitor); [4-[4-[2,4,6-trioxo-5-(4-pyrimidin-2-ylpiperazin-1-yl)hexahydropyrimidin-5-yl]phenoxy]phenyl]methyl N-(7-carbamimidoyl-1-naphthyl)carbamate.
9. AGB (Urokinase inhibitor); N-(1-adamantyl)-N'-(4-guanidino benzyl)urea
10. Pp3-3 [(2S)-3-[[(1S)-2-amino-1-(1H-indol-3-ylmethyl) - 2 - oxo - ethyl]amino] - 3 -oxo - 2 - [(3-phenylisoxazol-5-yl)methyl]propyl]-phenyl-phosphinic acid.
11. Pp3 3xAMR (hybrid inhibitor) [(2S)-3-[[(1S)-2-amino-1-(1H-indol-3-ylmethyl)-2-oxo-ethyl]amino]-2-[[3-[4- [[5- (carb amimidoyl carbamoyl)-3-chloro - pyrazin - 2 -yl] amino]phenyl] isoxazol-5-yl]methyl]-3-oxo-propyl]-phenyl-phosphinic acid.
12. Pp3 3xp4 4 (hybrid inhibitor) [(2S)-3-[[(1S)-2-amino-1-(1H-indol-3-ylmethyl)-2-oxo-ethyl]amino]-2-[[3-[4-[(7-carbamimidoyl-1-naphthyl)carbamoyloxymethyl]phenyl]isoxazol-5-yl] methyl] - 3 - oxo-propyl]-phenyl-phosphinic acid.

Conversion of two-dimensional to three-dimensional chemical structure.

When PDB 3D structure of chemicals existed it was used for molecular modeling and converted to PDBQT files through ADT. In some cases the ligand files were not in the proper format (SDF instead of PDB) or only a visual image of the structure was present. Files that were present in SDF format were converted to PDB using an online SMILES translator and structure file generator (https://cactus.nci.nih.gov/translate/). For visual models only, the inhibitors were built in 2D using Biovia draw (http://accelrys.com/). The 2D structure was then translated to a SMILES string and text was then translated by the online SMILES translator and structure file generator to the 3D PDB file. The PDB files generated through these alternative methods were then uploaded to ADT and converted to PDBQT files.

Protein structure preparation and Autodock analysis.

The structures of uPA (1F5L) (56) and MMP-9 (1GKC) (57) were downloaded as PDB files from RCSB Protein Data Bank. Each enzyme was open individually as a text file and the codes for water, bound ligands, and other compounds present in the file were deleted. Prior to deletion of the code, the coordinates of an individual atom in the center of a ligand (present in the active site of each enzyme) was recorded for later use. For urokinase the coordinates used were: x=30.502, y=6.741, z=28.432. For MMP-9 the coordinates used were: x=-0.135, y=22.280, z=13.282. The isolated enzymes were then uploaded to Autodock Tools (ADT). Using ADT, the coordinates and dimensions for the active sites of each enzyme were set. Urokinase active site size was set to 30 A on the x, y, and z axes, while for MMP-9 active site was set to 40 A on the all axes from the center defined by the above coordinates. Each enzyme was then saved as a PDBQT file as required for analysis by Autodock Vina.

Each PDBQT inhibitor file was analyzed using the Autodock Vina program which calculates the inhibitors affinity (kcal/mol) for a specified enzyme binding site. For each analysis Autodock Vina generated an output file with 9 potential 3D configurations of a ligand in an enzyme active site. Inhibitors were fitted in each enzyme and their respective output files were viewed in PyMol to ensure the best configuration was represented. The computed highest affinity as well as the observed best structure were considered as most probable final structure and corresponding affinity was recorded for each inhibitor.

$$K_i = exp\left(\Delta G \ / \ (R * T)\right)$$

where:
K_i is the inhibitory constant.
T is temperature in Kelvin (calculations done at 298K).
R is universal gas constant.

Generation and evaluation of hybrid molecules.

The inhibitors with the highest affinities for each enzyme were then used as templates for the production of a hybrid inhibitor (in this case a hybrid inhibitor refers to one that inhibits both Urokinase and MMP-9). The two inhibitors were bound through carbon-carbon, carbon-oxygen, or nitrogen-carbon bonds. The location of fusion of the two inhibitors aimed to leave the high affinity aspects of each on opposing ends of the new structure in order to maximize affinity for both urokinase and MMP-9 active sites. Structures were converted into PDBQT files and analyzed by Autodock Vina as described above.

Results and Discussion

To validate the Vina Autodock docking protocol we redock the ligands of urokinase (amiloride and p-aminobenzamidine) to crystallographic protein structure after removing ligands. Ligands with lowest free energy or highest calculated affinity were used for comparison. It is considered that a docking protocol should give RMSD < 2.0 A of crystallographic structure and that cutoff is frequently used as a criterion of the correct bound structure prediction (53). As it can be seen in Fig. 1 amiloride binds closely to its structure determined by X-ray crystallography (56). P-aminobenzamidine showed similarities to its crystal structure and RMSD where below 2 A for these two controls as determined in this study and in our previous work (data not shown) (25, 56, 58, 59).

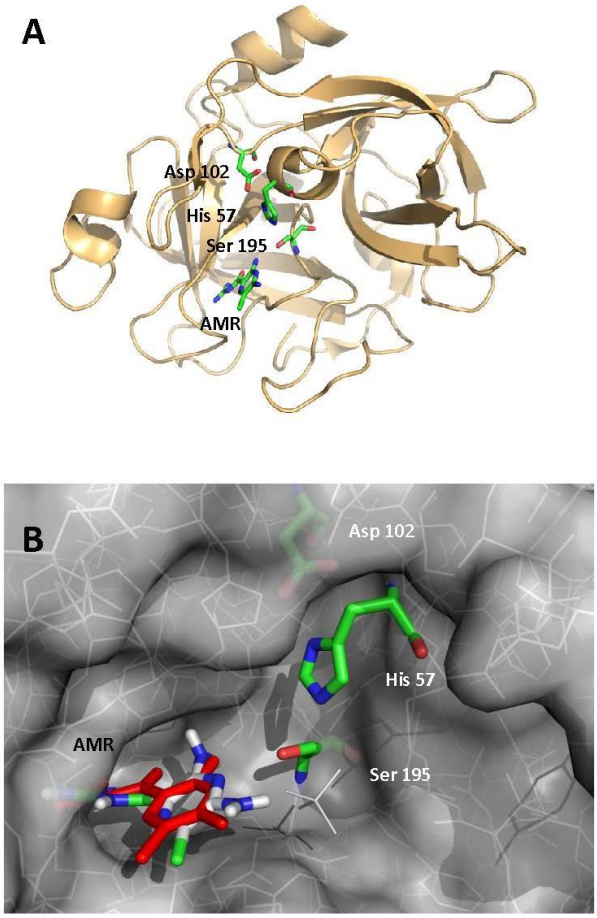

Fig. 1. A: carton model of urokinase (1F5L), amino acids of catalytic triad (His57, Asp 102 and Ser 195) are shown as sticks model and colored: carbon in green, oxygen in red, nitrogen in blue. B: surface of uPA is shown in semitransparent gray, amilorides position in specificity pocket are shown as stick model and colored: amiloride from crystallographic structure in red, best model calculated by Vina Autodock colored as amino acids. Only hydrogens of amiloride calculated by Vina Autodock are shown for clarity.

After testing of 21 potential inhibitors eight hybrid inhibitors were created from the best inhibitors and analyzed in silico. We have found that all the hybrids created had higher affinities for urokinase and MMP-9 than the control inhibitors (amiloride and STN) as can be seen in Table 1. The calculated affinity for amiloride bind to uPA was -7.8 kcal/mol while amiloride affinity bind to MMP-9 was -5.3 kcal/mol. The best hybrid (UI1xAGB) had affinities of -12.1 kcal/mol (or Ki = 1.61^{-9} mol) for MMP-9 and -12 kcal/mol ((or Ki = 1.36^{-9} mol)) for urokinase (Fig. 2). Analyzing the binding of each individual hybrid in the target enzymes through PyMol demonstrates the potential efficacy of each hybrid. Each hybrid binds to, or in close proximity to, the catalytic triad of the urokinase active site, and the catalytic zinc and corresponding histidine residues of the MMP-9 active site. Binding this way makes the enzymes inaccessible to other potential ligands resulting in the effective inhibition of the catalytic and/or metastatic activity of these enzymes.

Table 1. Calculated affinity for proteins inhibitors complexes shown as kcal/mol or as K_i

Inhibitor	MMP-9[a]	MMP-9[b]	uPA[a]	uPA[b]
ab14519	-10.2	3.35^{-8}	-6.5	2.42^{-5}
AGB	-9.9	5.57^{-8}	-8.2	9.81^{-7}
Ul1	-9.6	9.24^{-8}	-8.5	5.91^{-7}
7lN	-8.7	4.22^{-7}	-7.5	3.19^{-6}
AMRxab145190	-8.5	5.91^{-7}	-9.5	1.08^{-8}
2AMRxab145190	-8.9	4.22^{-7}	-9.7	7.81^{-8}
UI1xAGB	-12.1	1.36^{-9}	-12.0	1.61^{-9}
UI1xAMR	-10.1	3.97^{-8}	-8.6	4.09^{-7}
7INxAMR	-10.3	2.83^{-8}	-8.5	5.99^{-7}
Pp3 3	-10.0	1.29^{-7}	-9.4	8.28^{-7}
Pp3 3xAMR	-10.6	1.71^{-8}	-10.8	1.22^{-8}
Pp3 3xp4 4	-10.5	2.83^{-8}	-9.7	7.81^{-8}

affinity in a: kcal/mol, b: Ki mol.

During the process of binding and generation of 3D structures in silico there is variance in the affinity scores as well as 3D structure orientation. A test done multiple times will almost never generate identical results. This variance can be attributed to the programs attempt at an authentic binding simulation. When running a binding analysis, the program attempts to imitate the random motion of a ligand about the binding site coordinates that have been assigned. By doing so, each test results in different affinities, but the differences are so small that they are negligible.

Moving forward, each individual hybrid would need to be successfully synthesized for in vitro analysis in the lab. The newly synthesized hybrids would be tested using ligand binding assays to determine the degree of affinity, equilibrium constant, reliability and validity of linked reactions, etc. Further tests would need to be run to test the hybrids ability to effectively inhibit the target enzymes function as well as other potential interactions with non-target enzymes. Trials with animals induced with metastatic tumors would allow insight into the toxicity of the hybrid as well as its ability to control metastasis. From there, the goal would be clinical trials where it would hopefully be deemed safe and effective enough for commercial use against cancer metastasis.

Numerous in vivo and in vitro studies have demonstrated that inhibition of proteolytic activity can reduce cancer invasion, tumor size and limit angiogenesis (59-63). Consequently human clinical studies were designed inhibiting urokinase or MMPs, but these

target specific inhibitors producing mixed results (64-67). One of the possible explanations is that cancers are overexpressing at least urokinase and MMPs simultaneously (5, 68-70). Thus upregulated net proteolytic activity should be normalized rather that inhibiting single proteolytic enzyme.

Conclusion

Therapy of the malignances preventing invasion, metastasis and pathological angiogenesis should include downregulation of the variety of proteolytic enzymes. Creating bifunctional inhibitors of urokinase and metalloproteinase could provide an alternative to existing anticancer therapies.

Conflict of interest

Authors declare no conflict of interest.

Authors' contributions

SPB, JJ conceived and designed the experiments; SPB performed the calculations and formal analysis; JJ reviewed and revised the manuscript. Both authors have wrote the manuscript, read and approved the final document.

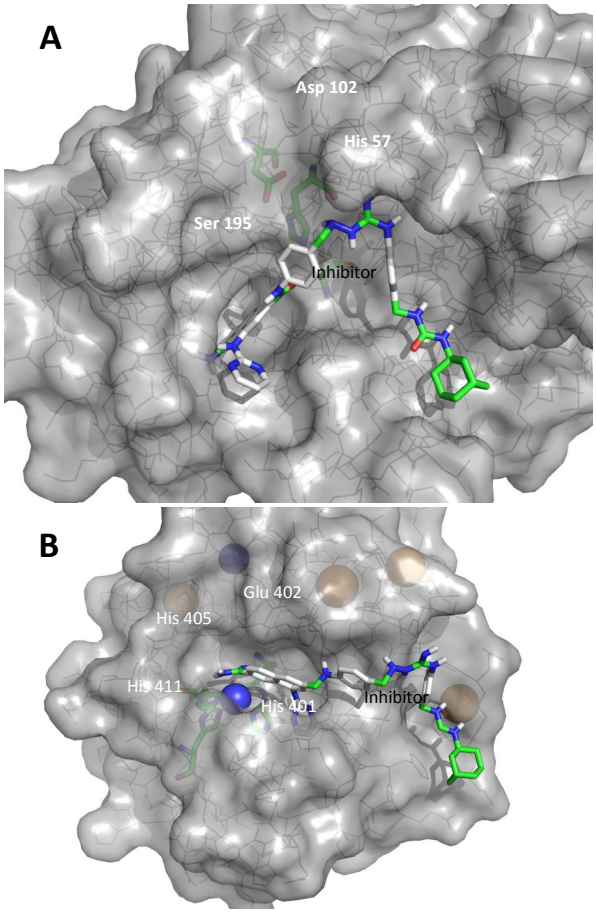

Fig 2. Best inhibitor of urokinase and MMP-9 (UI1xAGB). A: urokinase surface amino acids of catalytic triad (His57, Asp 102 and Ser 195) are shown as sticks model and colored: carbon i green, oxygen in red, nitrogen in blue, surface of uPA is shown in semitransparent gray. B: the catalytic center of MMP-9 is composed of the active-site zinc ion (shown as blue sphere), co-ordinated by three histidine residues (401, 405 and 411) and the essential glutamic acid residue (402) shown as a stick model colored: carbon - green, oxygen - red, nitrogen - blue), surface of uPA is shown in semitransparent gray.

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