Identification of potential small-molecule protein-protein inhibitors of cancer metastasis by 3D epitope-based computational screening

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Abstract

In cancer cells exposed to extracellular pressure or shear stress, AKT1-FAK interaction drives FAK phosphorylation, leading to force-activated cancer cell adhesion and metastasis. Blocking the AKT1-FAK interaction is therefore an attractive target for cancer therapy, avoiding the side effects of global FAK inhibition. Starting with our previous identification of a short FAK peptide that binds AKT1, we identified a series of small-molecule inhibitor candidates using a novel approach for inhibiting protein-protein interactions. Using a 3D structural fragment of the FAK peptide as the query, millions of drug-like, commercially available molecules were screened to identify a subset mimicking the volume and chemistry of the FAK fragment to test for their ability to block pressure-sensitive FAK phosphorylation by AKT1. Two compounds reduced the stimulation of FAK phosphorylation in response to extracellular pressure in human SW620 colon cancer cells without affecting basal FAK phosphorylation. Thus, using a 3D protein interaction epitope as a novel query for ligand-based virtual screening can successfully identify small-molecules that show promise in modulating cancer cell adhesion and metastasis.

Key words: Adhesion, cancer, ligand-based virtual screening, epitope mimic, FAK, metastasis

Highlights

- A 3D alignment and scoring method developed to discover small-molecule mimics of known small ligands has been newly applied to discover drug-like mimics of an epitope essential for kinase-kinase interaction.
- Two small-molecule mimics of the AKT1-binding FAK epitope were block force-activated cancer cell adhesion associated with metastasis.

1. Introduction

Most patients with cancer die not of their primary tumor but of metastatic disease, yet most cancer pharmacotherapy targets primary tumors and metastasis equivalently. Physical forces such as extracellular pressure [1] and shear [2] stimulate a complex cytoskeletally-dependent pathway within cancer cells that ultimately increases beta1 integrin heterodimer binding affinity, potentiating metastatic cancer cell adhesion.[3, 4] The effect lasts for up to an hour after force activation *in vitro*. Force-activated adhesion occurs in diverse malignancies, including colon and breast adenocarcinoma [5, 6], head and neck squamous cell cancer [7], and sarcoma [8]. Cancer cells shed during surgery may be activated by laparoscopic pressures, surgical manipulation, postoperative intra-abdominal pressure increases, or pressure and shear within the lymphatics or circulation. Blocking the pressure-activated pathway that stimulates cancer cell adhesion with high doses of colchicine [9] or siRNA to alpha-actinin-1 [10] improves survival in mouse models of postoperative local tumor recurrence. Blocking this pathway in surgical patients might reduce perioperative tumor dissemination and improve cancer survival.

A key step in the pathway involves AKT1 binding to focal adhesion kinase (FAK), and serine phosphorylation of FAK by AKT1 is required for FAK-Tyr-397 tyrosine autophosphorylation and subsequent FAK activation.[11] AKT1-FAK interaction is not generally required for FAK activation by other stimuli [12], while pressure-stimulated signals in macrophages depend upon AKT2, not AKT1 [13]. AKT1-FAK interaction therefore seems a target for intervention to prevent pressure-induced metastasis without inhibiting essential functions. We previously identified a 33-residue peptide within FAK that is required for FAK-AKT interaction [14] and reported that adenoviral overexpression of a 7-residue segment prevents AKT-FAK interaction and consequent force-activation of FAK, ultimately improving tumor-free survival in mice [15].

However, developing peptides as pharmaceuticals is challenging because mammalian digestion and serum proteases inactivate peptides. Blocking protein-protein interactions (PPIs) with drug-like molecules is challenging because PPI interfaces tend to be large and flat, making it hard for small molecules to make sufficient high-affinity contacts with one protein to block binding by its protein partner [16]. Consequently, PPI inhibitor discovery through screening by docking often has high false positive and negative rates [17]. One promising approach is to experimentally define and mimic an epitope containing several contiguous residues in one of the proteins that accounts for a significant portion of the PPI binding affinity [18]. Identifying the 7-residue FAK peptide described above is an example of this approach.

We propose and test a way to go a step further to discover *drug-like* inhibitors of PPIs. Three-dimensional ligand-based screening is typically used to discover new classes of drug-like mimics of a known bioactive small molecule (e.g., a substrate or metabolite). Here, we employ ligand-based screening to discover small molecules that mimic an excised region of a PPI epitope. This technique is powerful because it can equally well be used to find mimics of discontinuous or continuous epitopes. An advantage of ligand-based screening is that knowledge of the molecular interactions between the binding partners is not required; structural knowledge is only required for part of one protein's epitope. Ligand-based screening can match or outperform molecular docking and routinely successfully identify inhibitors [19-21]. Another advantage of ligand-based screening is that sources of inaccuracy in docking (e.g., modeling detailed flexibility, electrostatics and solvent contributions) can be avoided by directly identifying mimics of a known bioactive molecule rather than modeling its interactions with a partner. Using this approach, the 7-residue peptide from FAK was used as a query molecule to identify small molecules with drug-like properties that mimic part of the peptide. These candidates were then tested for their ability to block FAK phosphorylation by AKT1 and reduce force-activated cancer cell adhesion.

2. Materials and Methods

2.1. Computational screening

We developed a ligand-based virtual screening protocol (Figure 1) to identify small-molecule mimics of a the FAK peptidyl epitope (LAHPPEE) and a more helical analog (AAHPSEE) that also binds AKT1 [15]. For LAHPPEE, we focused on the more rigid LAHPP region (helix-turn) as likely bearing a similar peptidyl structure to intact human FAK (NCBI accession number: NP_722560.1) [22]. The 3D atomic coordinates for residues 113-117 (LAHPP) were extracted from the crystal structure of FAK (PDB entry: 2al6) [23]. To consider side-chain flexibility, structures reflecting eight favorable alternative positions of Leu were created with backbone-dependent rotamer sampling [24] in PyMOL v. 1.8.2.2 (Schrödinger, LLC); no alternative favorable positions for His were identified. The N- and C-termini of the peptide structure were capped to a neutral state, reflecting their state within intact FAK. To enable chemical matching of polar atoms during screening, partial atomic charges were computed and assigned to the LAHPP structures using molcharge (QUACPAC v. 1.6.3.1; https://www.eyesopen.com/quacpac; OpenEye Scientific Software, Santa Fe, NM) with the AM1BCC force-field [25]. Following partial charge assignment, extra protons were removed from the C- and N-terminal nitrogens, and nitrogen charges were set to -0.55, mimicking their state within FAK at physiological pH.

The second query peptide, AAHPSEE (Figure 2A), is a two-site mutant of residues 113-119 in human FAK. In the wild-type structure, the 7-residue peptide consists of a helix terminus followed by a turn. Together they may form a continuous helical epitope upon interaction with AKT1. To test this possibility, the AAHPSEE sequence was designed as a peptide variant with greater helicity, based on the high helical propensity of Ala and the ability of Pro-Ser to form a less bent helix than Pro-Pro. Sequery [26] and Superpositional Structure Assignment [27] were used to evaluate the helicity of sequences matching AAHPSEE in the Protein Data Bank [15, 27, 28]. AAHPSEE was subsequently shown to effectively compete with FAK for binding to AKT1 [15]. For 3D ligand-based screening, the structure of the AAHPSEE query was built as an alpha-helix in PyMOL [29], with Ser modeled to match the wild-type Pro conformation. The structure was then energy-minimized with YASARA (http://www.yasara.org/minimizationserver.htm) [30], with charges and termini handled as above for LAHPP. These peptide structures were then used as queries to discover the most similar drug-like candidates for testing as potential inhibitors of FAK activation by AKT1 and pressure stimulation of cancer cell adhesion.

For screening, 3D structure files of 10,639,555 commercially available molecules with drug-like properties defined by the Rule of 5 [31] were downloaded from ZINC (http://zinc.docking.org) [32] in MOL2 format. To test the ability of molecules from ZINC to match the known conformation and charge distribution for LAHPP and AAHPSEE peptides, up to 200 favorable 3D conformations were generated for each ZINC molecule using default settings in Omega (version 2.4.1; https://www.eyesopen.com/omega; OpenEye Scientific Software, Santa Fe, NM) [33]. To identify structural mimics of the FAK peptide queries, the 3D structures of the drug-like molecular conformers were overlaid on the query molecules using ROCS (version 2.4.6; https://www.eyesopen.com/rocs; OpenEye Scientific Software, Santa Fe, NM)

[20]. The 3D overlays were assessed by TanimotoCombo scoring, which equally weighs volumetric and chemical similarity. After the top-500 LAHPP and AAHPSEE mimics were identified via ligand-based screening, we removed any that were categorized as pan-assay interference compounds, using the PAINS-Remover server (http://cbligand.org/PAINS/) [34].

Final prioritization of molecules for assays was based on visual inspection in PyMOL [29], evaluating the closeness of alignment between the rigid scaffold of the inhibitor candidate and the peptide backbone, and the chemical and volumetric similarity in contiguous, surface-accessible side chains in the peptide. Close analogs of one of the molecules discovered by 3D virtual screening, ZINC04085549 (Figure 2B), were also selected for assays (Figure 3) using the SwissSimilarity webserver [35] (http://www.swisssimilarity.ch) and the http://zinc.docking.org/search/structure search with the ZINC database to find the most shape and chemically similar structures to ZINC04085549. In total, eleven molecules were selected for assay (Figures 3 and 4), representing different scaffolds.

2.2. Cells and reagents

Human SW620 colon cancer cells from the American Tissue Culture Collection were cultured as described [5]. Chemical compounds from commercial suppliers (Tables 1 and 2) were at the highest available purity.

2.3. Extracellular pressure treatment

Extracellular pressure was increased by 15 mmHg over ambient pressure using a temperature and pressure-controlled box as described.[36]

2.4 FAK-Y397 western blotting

Cells were maintained at ambient or increased pressure in bacteriologic plastic dishes pacificated with heat-inactivated bovine serum albumin to prevent adhesion and avoid adhesion-associated background FAK activation. Cells were lysed in lysis buffer, resolved by 10% SDS-PAGE, transferred to nitrocellulose and blotted with antibody to Tyr-397-phosphorylated FAK (rabbit monoclonal ab81298, Abcam, San Francisco, CA, USA) and anti-rabbit 680 (LI-COR Inc. Lincoln, NE, USA), before quantitation using Kodak Scientific Imaging Systems 1D, V.3.5.4 [15]. Total FAK (Anti-FAK, clone 4.47 Merck, Darmstadt, Germany with secondary anti-mouse 800, LI-COR Inc. Lincoln, NE, USA) served as a loading control. FAK and Tyr-397-phosphorylated FAK western blots yielded doublets similar to those previously observed by others [37-40]. We quantitated both bands together.

2.5 Adhesion assay

We seeded 50,000 cells/well into 24 well plates precoated with collagen I (Sigma, St. Louis, MO, USA) at 37°C under ambient or 15 mmHg increased pressure as described [36]. After 30 minutes, non-adherent cells were washed away. Adherent cells were stained with MTS (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Madison, WI, USA) and plates were read at 490 nm.

2.6 Statistical analysis

All assays were performed within linear ranges. Data were normalized against ambient pressure controls treated with DMSO as a vehicle control, represented as $X \pm SE$, and analyzed by t-test seeking 95% confidence.

3 Results

The eleven molecules described in the Methods and Figures 2, 3, and 4 were tested in human SW620 colon cancer cells for their ability to prevent either stimulation of FAK phosphorylation or stimulation of adhesion to collagen.

3.1 FAK phosphorylation studies

Four structurally similar molecules to the FAK-derived sequence LAHPP (Table T1) were evaluated for their ability to prevent FAK activation by 15 mmHg increased extracellular pressure in human SW620 colon cancer cells. FAK-Tyr-397 phosphorylation was measured as an early step in FAK activation, as previously demonstrated in the pressure-activated adhesion pathway [5] and in FAK activation by other stimuli [41, 42]. Three molecules, ZINC31501681, ZINC58264388, and ZINC40099027 increased basal FAK-Tyr-397 phosphorylation even at ambient pressure (Table T1 and/or Figure 3). Each also prevented a further pressure-induced increase in FAK-Tyr-397 phosphorylation. At 1 nM, ZINC25613745 affected neither basal nor pressure-stimulated FAK-Tyr-397 phosphorylation. Figure 5 shows a typical study. The tendency to increase basal FAK phosphorylation made these LAHPP mimics unattractive for further study, given the goal of blocking FAK activation by phosphorylation.

3.2. Adhesion studies

We next studied seven molecules that structurally mimic AAHPSEE, including five analogs of ZINC04085549 (Figure 3). These molecules' effects on basal and pressure-stimulated human SW620 colon cancer cell adhesion to collagen I were assayed first. Most molecules had no effect at the concentrations studied (Table 2). However, two molecules, ZINC04085549 and ZINC4085554, prevented pressure-stimulated increases in SW620 adhesion without altering basal adhesiveness at ambient pressure (Figure 6).

3.3. Effects of ZINC04085549 on FAK-Tyr-397 phosphorylation

As one of the promising molecules preventing pressure-stimulated adhesion, ZINC04085549 was further evaluated for the ability to prevent pressure-stimulated FAK-Tyr-397 phosphorylation. At 50 μM, ZINC04085549 prevented pressure-stimulated FAK-Tyr-397 phosphorylation without affecting basal ambient pressure FAK-Tyr-397 phosphorylation (Figure 4).

4 Discussion

In vitro high-throughput screening of a large molecular library to identify compounds that block a transiently force-activated pathway would be challenging, because of the time-dependence of the effect and the cost of assaying many purified compounds. Instead, we took advantage of identification of a short FAK peptide involved in AKT1 binding to perform a large-scale computational screen for drug-like molecules mimicking the peptide. By assaying 11 top molecules from the screen, two dinitrobenzene containing molecules, ZINC04085549 and ZINC4085554 (Figure 3), were discovered that block the pressure-induced adhesion of cancer cells. While dinitrobenzene has an unfavorable chemical safety profile (https://www.cdc.gov/niosh/ipcsneng/neng0691.html), nitroglycerin exhibits similar hazards, https://www.cdc.gov/niosh/ipcsneng/neng0186.html), and yet is used to treat angina. Innocuous analogs of these compounds may affect FAK activation similarly. Otherwise, both compounds have drug-like features according to Lipinski's Rule of 5: ≤5 H-bond donors, ≤10 H-bond acceptors, molecular weight ≤500D, and predicted aqueous solubility, as estimated by clogP ≤5 [43].

Another important result is that a ligand-based screening technique developed originally to find mimics of small molecules can be effective and efficient in discovering drug-like molecules that mimic PPI epitopes, a much greater challenge. This approach may be applied to other therapeutically relevant PPIs, such as growth factor and immune receptors.

Some candidate molecules activated basal FAK phosphorylation. FAK-397 tyrosine phosphorylation is generally an autophosphorylation event that occurs when FAK dimerizes with itself or other proteins to induce a conformational shift that moves its inhibitory FERM domain and permits autophosphorylation [12]. Phosphorylation is maintained until FAK is dephosphorylated by a tyrosine phosphatase such as SHP2 [44] or PTEN [45]. This phosphorylation often occurs when FAK is localized in the focal adhesion complex either after integrin-matrix binding [46, 47], after transactivation by other extracellular signals such as muscarinic receptor occupancy [48] or in response to mechanical stimuli such as repetitive deformation or pressure [3, 7, 49]. After phosphorylation, FAK may be displaced from the focal adhesion complex into the cytosol where it can be dephosphorylated. How these candidate molecules intervened in this sequence to activate basal FAK awaits study.

In contrast, ZINC04085549 and ZINC4085554 did not affect basal FAK phosphorylation but prevented FAK phosphorylation and adhesion in response to extracellular pressure. Although this level of pressure-induced FAK activation seems modest, it causes substantial survival differences in mouse models of tumor implantation.[9]

Future work will test alternative functional groups in ZINC04085549 and ZINC4085554 to enhance their potency and safety. The results show that ligand-based virtual screening can quickly select from millions of compounds a small number of readily testable, commercially available compounds that are enhanced in activity against pressure-induced cancer cell adhesion, which has been a subtle and intractable problem in cancer therapy.

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Declaration of conflict of interest: None.

Figure Legends

Fig. 1. Flowchart outlines 3D ligand-based virtual screening for identifying small-molecule mimics of epitopes from FAK.

Fig. 2. (A) Molecular structure of the AAHPSEE peptidyl epitope used for screening (sticks), shown in place of the wild-type residues 113-119, LAHPPEE, of the FAK FERM domain (blue surface; PDB entry 2al6). (B) Structure of AHHPSEE (carbons in green) overlaid with ZINC04085549 (carbons in aqua), rotated by ~90° around the z-axis relative to the view in (A). (C) 2D structure of ZINC04085549 with other identifiers.

Fig. 3. AAHPSEE mimics tested. (A) Two small-molecule mimics (green) selected from ROCS overlays with AAHPSEE (yellow). (B) Molecules identified via ZINC 2D-Tanimoto similarity search (*) or SwissSimilarity electroshape search (**) using ZINC04085549 from (A) as the query.

Fig. 4. LAHPP mimics assayed. The drug-like mimics overlaid by ROCS are shown with carbons in green; LAHPP is overlaid with carbons in yellow.

Fig 5. ZINC31501681 enhances the phosphorylation of FAK-Tyr-397 in suspended human SW620 cells at ambient pressure. SW620 cells treated with 0.1% DMSO as a vehicle control or 1-100 pM ZINC31501681 were subjected to ambient (A) or 15 mmHG increased pressure (P) for 30 minutes. A depicts representative western blots probed for FAK-Tyr-397 phosphorylation or total FAK which served as a loading control. B summarizes the densitometric quantitation of pFAKY397/FAK from five independent experiments. (* denotes $p \leq 0.05$ for comparison between 0.1% DMSO ambient and pressure while # denotes $p \leq 0.05$ for comparisons between 0.1% DMSO and 1-100 pM ZINC31501681 at ambient pressure.)

Fig. 6. ZINC04085549 blocks stimulation of SW620 cell adhesion to collagen I by increased extracellular pressure. SW620 cells were treated with 0.1% DMSO (vehicle control) or ZINC04085549 at 10-100 µM and allowed to adhere to collagen I for 30 minutes at ambient or 15 mmHg increased pressure. (n=4, *$p \leq 0.05$)

Fig. 7. ZINC04085549 blocks pressure-stimulated phosphorylation of FAK-Tyr-397. Suspended SW620 cells treated with 0.1% DMSO (vehicle control) or 50 µM ZINC04085549 were incubated at ambient (A) or 15 mmHg increased pressure (P). A shows representative blots probed for FAK-Tyr-397 phosphorylation and total FAK. B summarizes densitometric quantitation of pFAKY397/FAK from five independent experiments. (*$p \leq 0.05$)

Table Legends

Table 1. Assays of four compounds that mimic the LAHPP epitope in FAK.

Table 2. Assays of seven compounds that mimic the active two-site FAK mutant peptide, AAHPSEE

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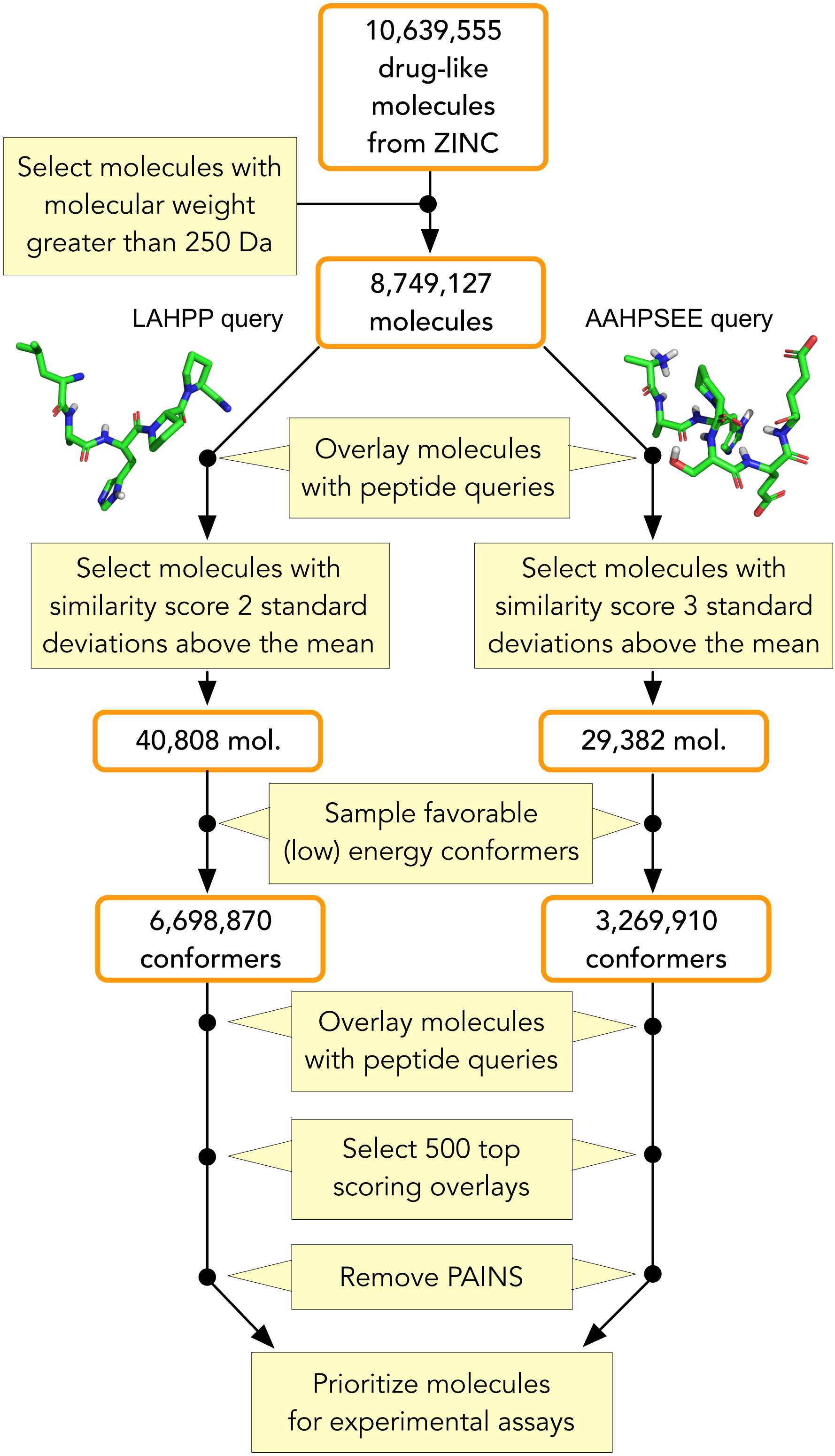
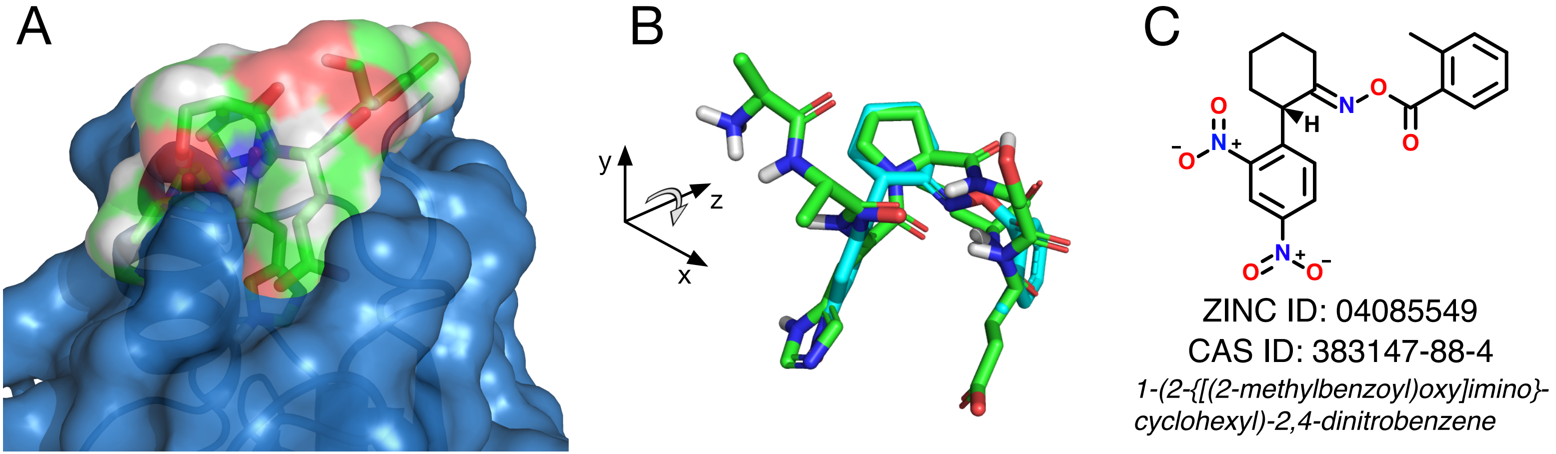


10,639,555 drug-like molecules from ZINC
Select molecules with molecular weight greater than 250 Da
8,749,127 molecules
LAHPP query
AAHPSEE query
Overlay molecules with peptide queries
Select molecules with similarity score 2 standard deviations above the mean
Select molecules with similarity score 3 standard deviations above the mean
40,808 mol.
29,382 mol.
Sample favorable (low) energy conformers
6,698,870 conformers
3,269,910 conformers
Overlay molecules with peptide queries
Select 500 top scoring overlays
Remove PAINS
Prioritize molecules for experimental assays


A
B
y
z
x
C
O
N
O
H
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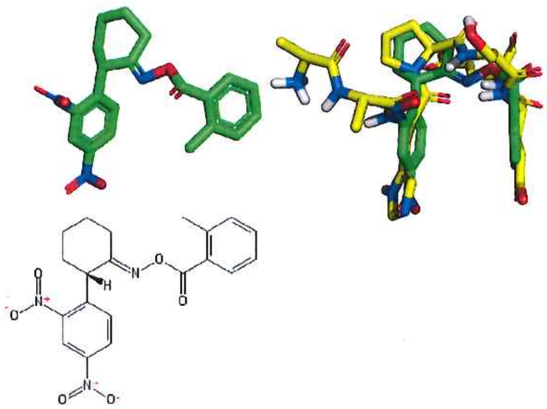
N
O
N
O
ZINC ID: 04085549
CAS ID: 383147-88-4
1-(2-{[(2-methylbenzoyl)oxy]imino}-
cyclohexyl)-2,4-dinitrobenzene

A

ZINC04085549

1-(2-{[(2-methylbenzoyl)oxy]imino}-cyclohexyl)-2,4-dinitrobenzene



ZINC02457454

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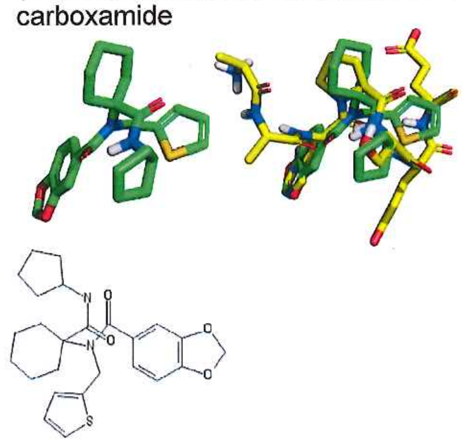

B

ZINC04085550*

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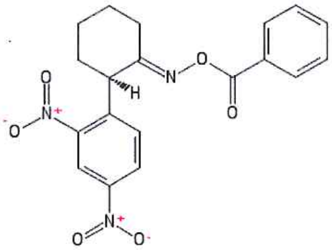
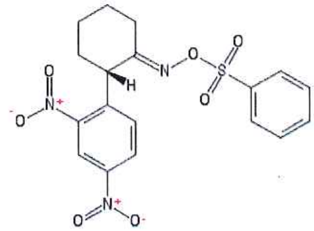


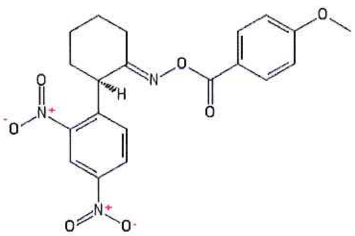
ZINC12960430*

N-[2-(2,4-dinitrophenyl)cyclohexyliden]-N-[(phenylsulfonyl)oxy]amine



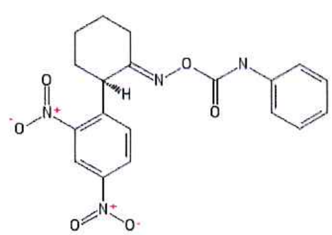
ZINC4085554**

1-(2-{[(4-methoxybenzoyl)oxy]imino} cyclohexyl)-2,4-dinitrobenzene



ZINC6241139**

1-(3-{[(anilinocarbonyl)oxy]imino} cyclohexyl)-2,4-dinitrobenzene

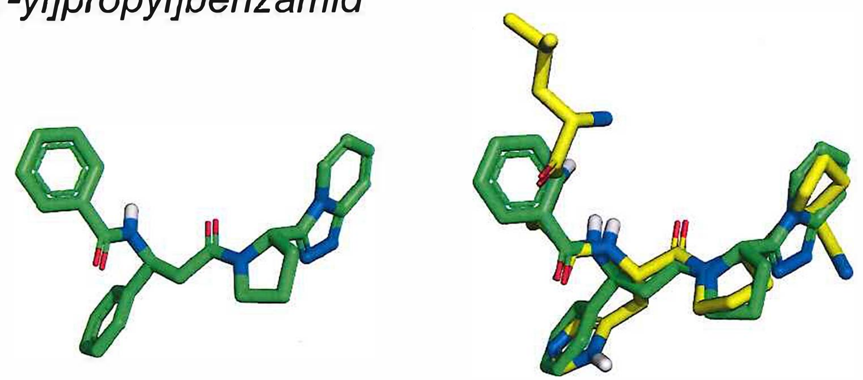


ZINC5816335**

1-(2-{[(cyclopentylcarbonyl)oxy]imino} cyclohexyl)-2,4-dinitrobenzene

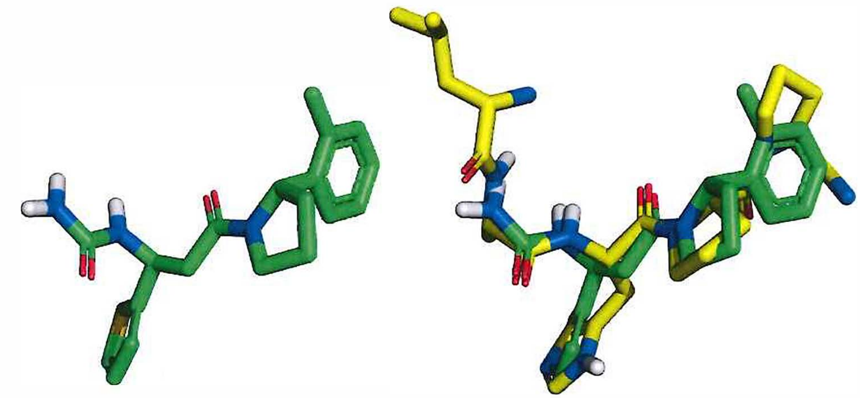
ZINC31501681

N-[(1S)-3-oxo-1-phenyl-3-[(2S)-2-([1,2,4]triazolo[4,3-a]pyridin-3-yl)pyrrolidin-1-yl]propyl]benzamid



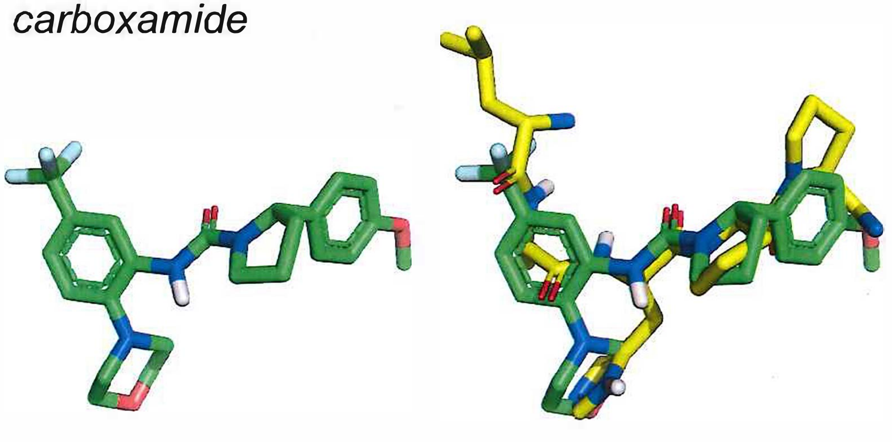
ZINC58264388

[(1S)-3-[(2S)-2-(o-tolyl)pyrrolidin-1-yl]-3-oxo-1-(2-thienyl)propyl]urea



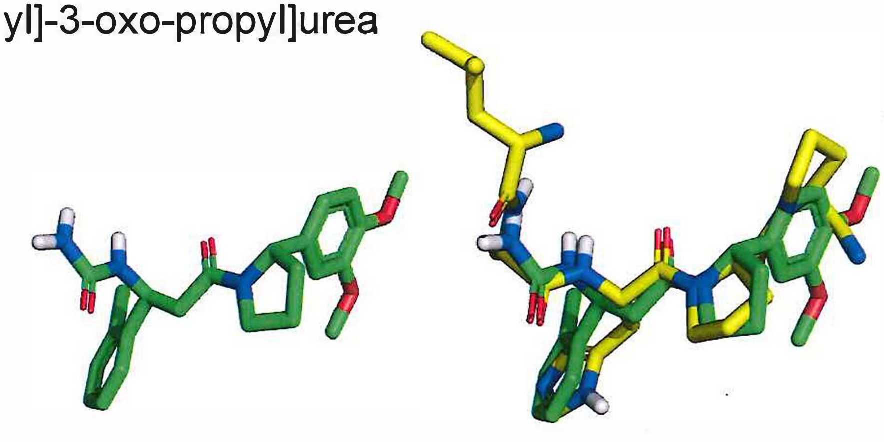
ZINC40099027

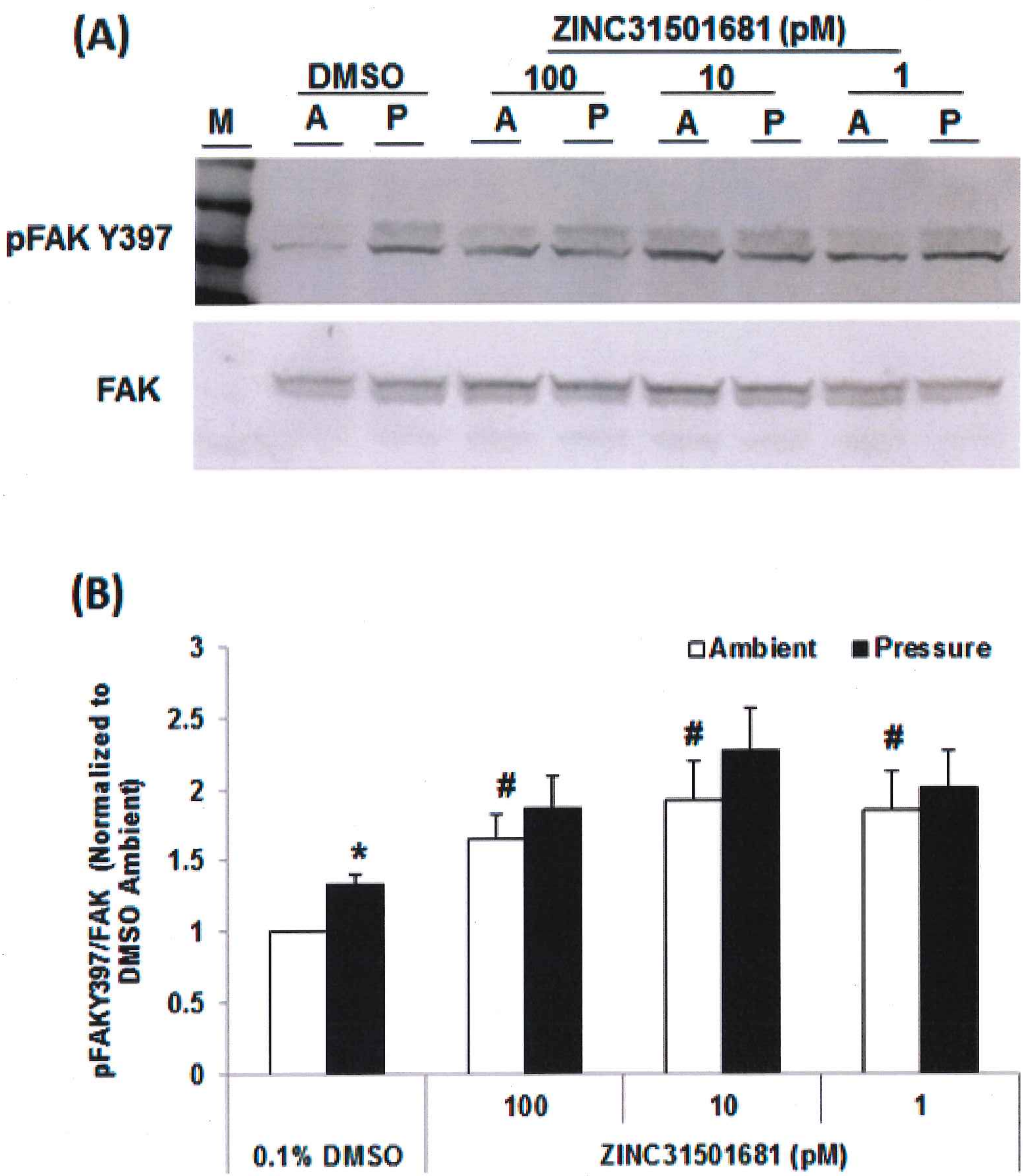
(2S)-2-(4-methoxyphenyl)-N-[2-morpholino-5-(trifluoromethyl)phenyl]pyrrolidine-1-carboxamide



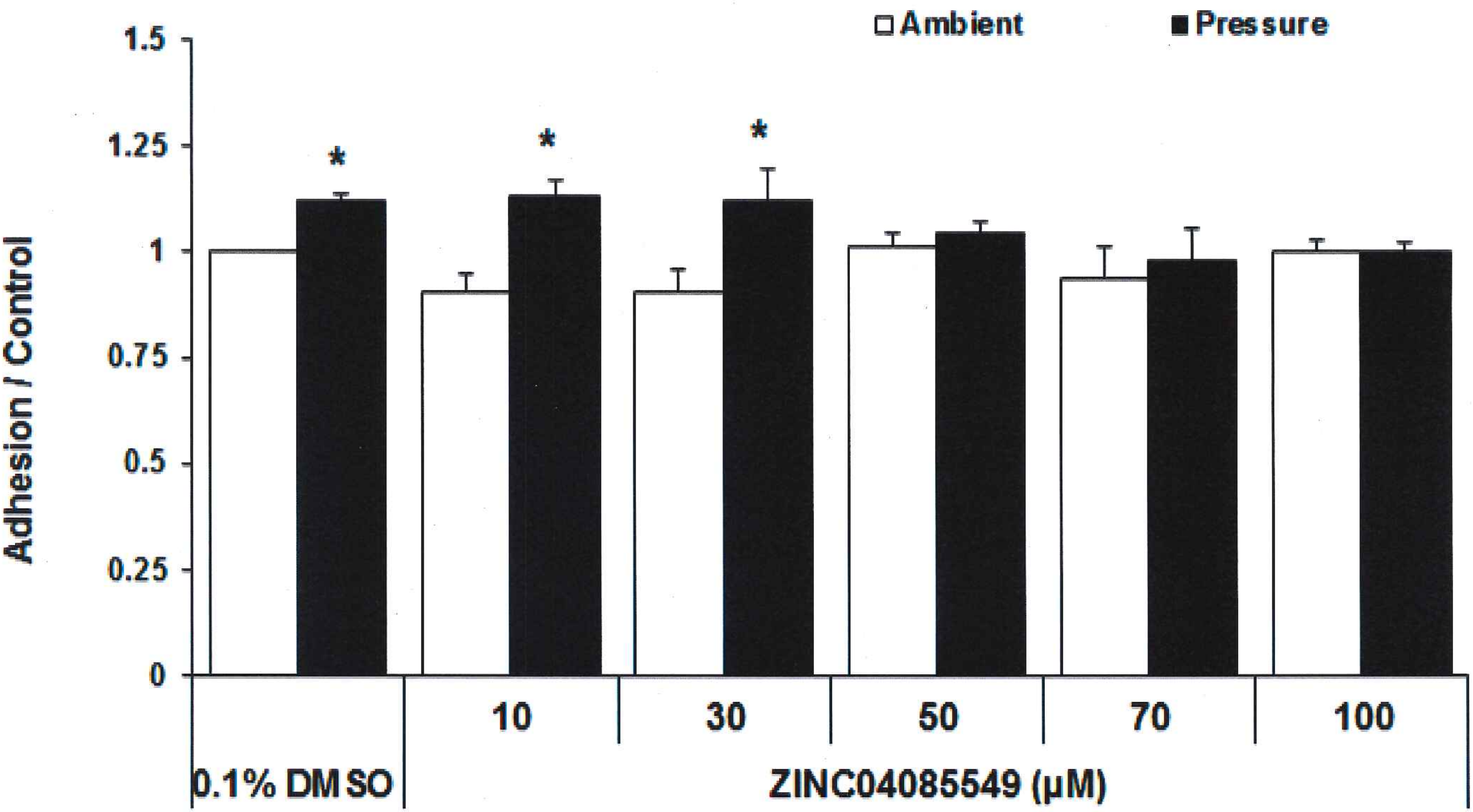
ZINC25613745

[(1S)-1-(2-chlorophenyl)-3-[(2S)-2-(3,4-dimethoxyphenyl)pyrrolidin-1-yl]-3-oxo-propyl]urea

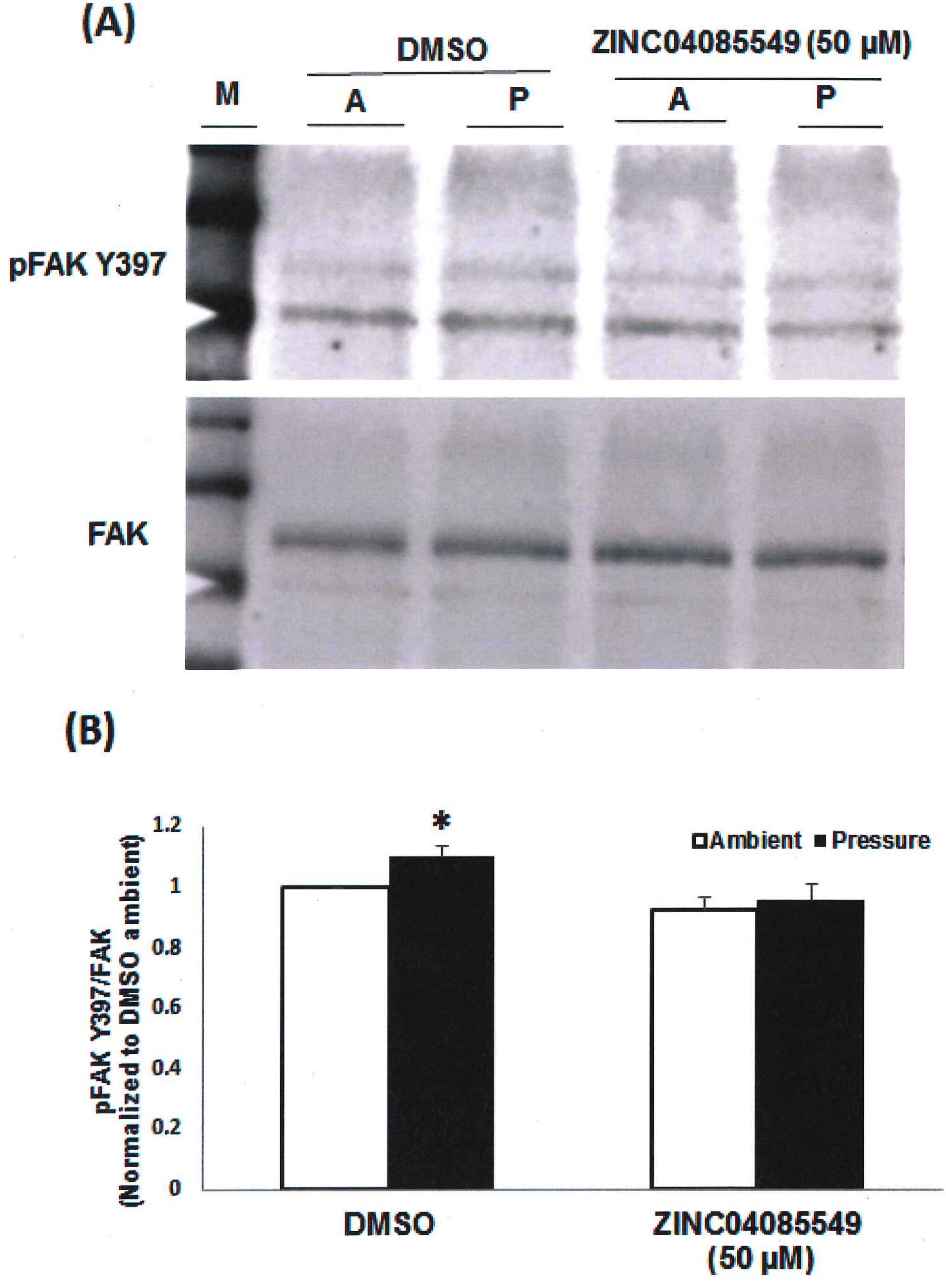




(A)
ZINC31501681 (pM)
DMSO
100
10
1
M
A
P
A
P
A
P
A
P
pFAK Y397
FAK
(B)
Ambient
Pressure
pFAKY397/FAK (Normalized to DMSO Ambient)
3
2.5
2
1.5
1
0.5
0
*
#
#
#
100
10
1
0.1% DMSO
ZINC31501681 (pM)



Ambient
Pressure
Adhesion / Control
1.5
1.25
1
0.75
0.5
0.25
0
*
*
*
10
30
50
70
100
0.1% DMSO
ZINC04085549 (μM)


(A)
DMSO
ZINC04085549 (50 μM)
M
A
P
A
P
pFAK Y397
FAK
(B)
pFAK Y397/FAK (Normalized to DMSO ambient)
1.2
1
0.8
0.6
0.4
0.2
0
*
Ambient
Pressure
DMSO
ZINC04085549 (50 μM)

ZINC name	Concentrations studied	Basal FAK-pTyr397	Pressure stimulation of FAK-pTyr397	Vendor/Supplier
ZINC31501681	1 pM - 300 μM	Increased	No further increase	FCH Group (Made to order), Chernigov, Ukraine
ZINC58264388	1 nM – 100 nM	Increased (10-100 nM)	No further increase (10-100 nM)	ENAMINE Limited, Kiev, Ukraine
ZINC40099027	10 pM - 10 nM	Increased	No further increase	ENAMINE Limited, Kiev, Ukraine
ZINC25613745	1 nM	No change	Maintained	ENAMINE Limited, Kiev, Ukraine

ZINC name	Concentrations studied	Basal adhesion	Pressure stimulated adhesion	Vendor/Supplier
ZINC04085549	10-100 μM	Maintained	Blocked ≥50 μM	BIONET/Keyorganics Limited, Bedford, MA, USA
ZINC02457454	10-200 μM	Maintained	Not inhibited	ChemDiv, Inc. Vistas-M Laboratory, Ltd. (Premium), San Diego, USA
ZINC04085550	50 μM	Maintained	Not inhibited	BIONET/Keyorganics Limited, Bedford, MA, USA
ZINC12960430	50 μM	Maintained	Not inhibited	BIONET/Keyorganics Limited, Bedford, MA, USA
ZINC4085554	10-50 μM	Maintained	Blocked ≥50 μM	BIONET/Keyorganics Limited, Bedford, MA, USA
ZINC6241139	50 μM	Maintained	Not inhibited	BIONET/Keyorganics Limited, Bedford, MA, USA
ZINC5816335	10-50 μM	Maintained	Not inhibited	BIONET/Keyorganics Limited, Bedford, MA, USA