Potent inhibitors of human LAT1 (SLC7A5) transporter based on dithiazole and dithiazine compounds for development of anticancer drugs

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ABSTRACT

The effect of dithiazole and dithiazine compounds on the human (h)LAT1 transporter has been screened in proteoliposomes. Inhibition was tested on the antiport catalysed by hLAT1 as transport of extraliposomal [^{3}H]histidine in exchange with intraliposomal histidine. Out of 59 compounds tested, 8 compounds, showing an inhibition higher than 90% at 100 µM concentration, were subjected to dose-response analysis. Two of them exhibited IC_{50} lower than 1 µM. Inhibition kinetics, performed on the two best inhibitors, resulted in a mixed type inhibition with respect to the substrate. Furthermore, inhibition of the transporter was still present after removal of compounds from the reaction mixture, but was reversed on addition of dithioerythritol, a S-S reducing agent, indicating the involvement of disulfide(s) between the compounds and the protein. Molecular docking of the two best inhibitors on the hLAT1 homology structural model, highlighted interaction with the substrate binding site and formation of a covalent bond with the residue C407. Indeed, the inhibition was impaired in the hLAT1 mutant C407A confirming the involvement of that Cys residue. Treatment of SiHa cells expressing hLAT1 at relatively high level, with the two most potent inhibitors led to cell death which was not observed after treatment with a compound exhibiting very poor inhibitory potency.

Abbreviations: LAT, L-type amino acid transporters 1; SLC, Solute carrier; CD98, cluster of differentiation 98; ASCT2, AlaSerCys Transporter 2; mTOR, mammalian target of rapamycin; BBB, blood brain barrier; AdiC, L-arginine/agmatine antiporter, $C_{12}E_8$, octaethylene glycol monododecyl ether; BCH, 2-amino-2-norbornanecarboxylic acid; DTE, dithioerythritol; TX-100, Triton X-100

Keywords: LAT1 inhibitors; 1,2,3-dithiazoles; 1,2,4-dithiazines; mechanism of inhibition; pharmacological target; cancer.

1. Introduction

LAT1 (SLC7A5) is a branched chain and aromatic amino acid transporter belonging to the Heterodimeric Amino acid Transporter group (HATs). Structurally, it is characterized by the association of the light chain LAT1 with the glycoprotein CD98 (SLC3A2). The interaction between the two proteins occurs through a conserved disulfide bond between C164 and C109 of the human isoforms of LAT1 and CD98, respectively [1]. The structure of CD98 has been solved by X-ray crystallography [2] while that of LAT1 has been only predicted by homology modeling [3, 4]. Recently, LAT1 was demonstrated to be the sole transport competent subunit, able to recognize His as one of the major substrates. Transport is not dependent on sodium gradient or on pH [3, 5]. LAT1 gives rise to an antiport reaction that follows a random simultaneous mechanism in which amino acids bind with no preferential order to the transporter. The substrate binding site is characterized by the key residues F252, S342, C335 and C407. In particular, F252 is the gate element that allows substrate entry, while S342 and C335 are involved in substrate binding prior to translocation [3]. LAT1 is localized in several body districts among which, the Blood Brain Barrier where it regulates the exchange of eight out of nine essential amino acids. Alteration of this function, was recently shown to cause Autism Spectrum Disorders [5]. Concerning its sub-cellular localization, LAT1 is mainly expressed at the plasma membrane but its presence in lysosomal membrane has also been reported [6]. In this organelle, the transporter may play a role in cell signaling, as demonstrated for SLC38A9 which transduces Gln and Arg sufficiency to mTORC1 [7, 8]. Nevertheless, trafficking of LAT1 to these alternative cell localizations remains to be better defined.

The biomedical and pharmacological interest on LAT1 resides in its over-expression in many tumors [9]. In fact, due to its specificity also for Gln, it is involved in “Glutamine addiction”, a typical feature of tumor cells that use Gln for energy purpose at a much higher rate than normal cells. In this respect, LAT1 realizes a Gln/Leu cycle in concert with another transporter, namely ASCT2 that is a Na^+-dependent antiporter for Gln and other neutral amino acids. Therefore, both

transporters are involved in cancer growth and progression [10]. LAT1 is, indeed, considered as a marker of malignancy [9, 11, 12] and as a new pharmacological target [13, 14]. Several research groups worldwide are involved in the search for new molecular entities to be used as inhibitors of LAT1. The most common approaches used to achieve this goal are: i) *in silico* virtual screening that, however, requires high quality three dimensional structure of the target and ii) *in vitro* screening, generally performed with cell models. Recently, we exploited proteoliposome technology for assaying LAT1 function. With this model, the activity of a membrane protein can be detected in a "clean" system without interferences by other transport or enzymatic systems. Using this technology, a screening of a large group of potential inhibitors with dithiazole- or dithiazine-based structure was performed. Dithiazoles were previously tested on the rat isoform of ASCT2 and some lead molecules were identified exhibiting IC_{50} from 4 to 10 μM. Interestingly, the hypothesized molecular mechanism of ASCT2 inhibition was based on reaction of dithiazoles with specific Cys residue(s) of the protein, belonging to a CXXC motif of the rat protein [15, 16]. This type of covalent reaction mechanism was exploited for stably inactivating LAT1. The results obtained in the present study led to the identification of potent hLAT1 inhibitors.

2. Materials and methods

2.1. Materials

His Trap HP columns and PD-10 columns were purchased from GE Healthcare; radiolabeled amino acids were purchased from ARC (American Radiolabeled Chemicals); all the other reagents are from Sigma-Aldrich.

*2.2. Synthesis of 1,2,3-dithiazoles **1-55** and 1,2,4-dithiazines **56-59***

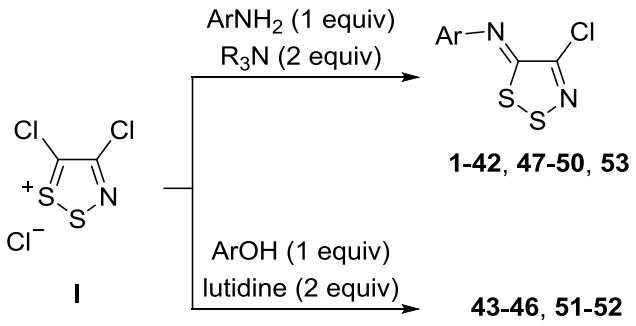
2.2.1. General methods and materials

Reactions were protected from atmospheric moisture by $CaCl_2$ drying tubes. Anhydrous Na_2SO_4 was used for drying organic extracts, and all volatiles were removed under reduced pressure.

Decomposition points were determined using the TA Instrument DSC Q1000 with samples hermetically sealed in aluminium pans under an argon atmosphere, using heating rates of 5 °C/min. UV/vis spectra were obtained using a Shimadzu UV-1601 UV/vis spectrophotometer and inflections are identified by the abbreviation "inf". IR spectra were recorded on a Shimadzu FTIR-NIR Prestige-21 spectrometer with a Pike Miracle Ge ATR accessory. NMR spectra were recorded on a Bruker Avance 500 MHz instrument. Deuterated solvents were used for homonuclear lock and internal calibration. MALDI-TOF mass spectra were recorded on a Bruker Autoflex III Smartbeam instrument. Elemental analysis was performed on a Perkin Elmer 2400 Series Elemental Analyzer by Stephen Boyer of London Metropolitan University.

*2.2.2. Synthesis of 1,2,3-dithiazoles **1-55***

For the preparation of the 1,2,3-dithiazoles, primary aromatic or heteroaromatic amines or alcohols were reacted with 4,5-dichloro-1,2,3-dithiazolium chloride (Appel's salt) I, followed by treatment with base (2 equiv) to give the corresponding [(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]arenes or 2-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)arene-1(2*H*)-ones **1-53** in moderate to excellent yields [17-19] according to the following general procedure: to a stirred suspension of Appel's salt I (100 mg, 0.48 mmol) in dichloromethane (4 mL) at *ca.* 20 °C was added the corresponding aminoarene or hydroxyarene (0.48 mmol). After 1 h, the appropriate base [*e.g.* Hunig's base (167 µL, 0.96 mmol)] was added dropwise to the reaction mixture. After stirring of 2 h the reaction mixture was loaded onto silica and purified by dry flash chromatography to afford the corresponding [(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]arene or 2-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)arene-1(2*H*)-one.



Analytical and spectroscopic data of the 1,2,3-dithiazoles **1-46** and **51-53** have been previously reported [15]. Analytical, physicochemical and spectroscopic data of the newly synthesized 1,2,3-dithiazoles **47-50** are reported below:

*(*Z*)-4-Chloro-*N*-[4-(morpholinomethyl)phenyl]-5*H*-1,2,3-dithiazol-5-imine (**47**).* Yellow oil (66%); R_f 0.35 (DCM/Et_2O, 70:30); (found: C, 47.59; H, 4.38; N, 13.01. $C_{13}H_{14}ClN_3OS_2$ requires C, 47.62; H, 4.30; N, 12.82%); λ_{max}(DCM)/nm 240 inf (log ε 4.16), 305 (3.44), 378 (3.84), 387 inf (3.83), 410 inf (3.72); ν_{max}/cm^{-1} 2955w, 2853w and 2805m (alkyl C-H), 1582m, 1533w, 1497m, 1452m, 1414w, 1395w, 1368w, 1348m, 1333m, 1308w, 1292m, 1263m, 1221m, 1115s, 1069m, 1034m, 1007m, 914m, 866s, 797m, 764m; δ_H(500 MHz, acetone-d_6) 7.49 (1H, d, *J* 8.2), 7.23 (1H, d, *J* 8.4), 3.63 (2H, t, *J* 4.7), 3.55 (1H, s), 2.44 (3H, br s); δ_C(125 MHz, acetone-d_6) 158.8 (s), 150.9 (s), 148.7 (s), 137.3 (s), 131.4 (d), 120.4 (d), 67.4 (t), 63.3 (t), 54.4 (t); *m/*z (MALDI-TOF) 328 (M^++1, 41%), 326 (M^+-1, 100).

*(*Z*)-4-Chloro-*N*-[3-(morpholinomethyl)phenyl]-5*H*-1,2,3-dithiazol-5-imine (**48**).* Yellow oil (81%), decomp. (DSC) onset: 145.6 °C, peak max: 150.5 °C; R_f 0.48 (DCM/Et_2O, 70:30); (found: C, 47.70; H, 4.20; N, 12.94. $C_{13}H_{14}ClN_3OS_2$ requires C, 47.82; H, 4.30; N, 12.82%); λ_{max}(DCM)/nm 238 inf (log ε 4.11), 302 (3.35), 377 (3.78), 386 inf (3.77), 409 inf (3.63); ν_{max}/cm^{-1} 2957w, 2855w and 2805m (alkyl C-H), 1578m, 1524w, 1503w, 1479m, 1454m, 1433m, 1396w, 1368w, 1348m, 1331m, 1300m, 1281m, 1261m, 1206w, 1163m, 1115s, 1069m, 1034m, 1009m, 908m, 885m, 856s, 806m, 785m, 758m; δ_H(500 MHz, $CDCl_3$) 7.41 (1H, dd, *J* 7.7, 7.7), 7.26–7.15 (2H, m), 7.11 (1H, d, *J* 7.8), 3.71 (5H, t, *J* 4.5), 3.53 (2H, s), 2.46 (4H, br s); δ_C(125 MHz, $CDCl_3$) 158.6 (s), 151.2 (s),

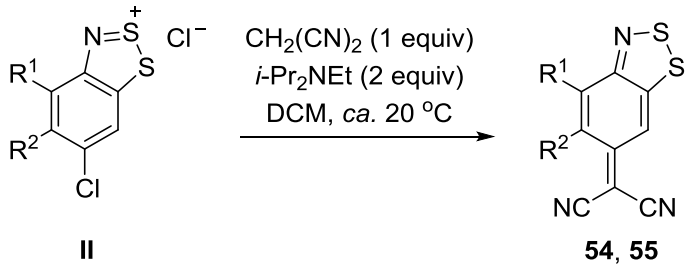
147.9 (s), 140.0 (s), 129.7 (d), 127.3 (d), 120.0 (d), 118.1 (d), 67.0 (t), 63.1 (t), 53.6 (t); *m/*z (MALDI-TOF) 328 (MH^+, 36%), 326 (M^+-1, 100), 292 (2), 260 (23), 233 (6), 153 (3).

*(*Z*)*-N-*Benzyl-1-{3-[(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]phenyl}*-N-*methylmethanamine* ***(49)****.* Yellow oil (86%), decomp. (DSC) onset: 145.3 °C, peak max: 154.1 °C; R_f 0.47 (DCM/Et_2O, 90:10); (found: C, 56.49; H, 4.38; N, 11.73. $C_{17}H_{16}ClN_3S_2$ requires C, 56.42; H, 4.46; N, 11.61%); λ_{max}(DCM)/nm 241 inf (log ε 4.17), 316 inf (3.45), 378 (3.83), 388 inf (3.82), 409 inf (3.69); ν_{max}/cm^{-1} 3059w and 3026w (aryl C-H), 2833w and 2785m (alkyl C-H), 1578s, 1495m, 1479m, 1452m, 1415w, 1364m, 1250m, 1159m, 1132m, 1076w, 1026m, 988m, 951w, 934w, 905m, 887m, 856s, 787m, 760m; δ_H(500 MHz, $CDCl_3$) 7.41 (1H, dd, *J* 7.7, 7.7), 7.40–7.36 (2H, m), 7.33 (2H, dd, *J* 7.5, 7.5), 7.30–7.21 (3H, m), 7.11 (1H, d, *J* 8.0), 3.57 (2H, s), 3.56 (2H, s), 2.21 (2H, s); δ_C(125 MHz, $CDCl_3$) 158.5 (s), 151.2 (s), 148.0 (s), 141.4 (s), 138.9 (s), 129.7 (d), 128.9 (d), 128.3 (d), 127.1 (d), 127.0 (d), 119.7 (d), 118.0 (d), 61.9 (t), 61.4 (t), 42.2 (q); *m/*z (MALDI-TOF) 362 (MH^+, 31%), 360 (M^+-1, 100), 294 (17), 267 (18), 91 (1).

*(*Z*)*-N-*Benzyl-1-{4-[(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]phenyl}*-N-*methylmethanamine* ***(50)****.* Yellow oil (90%), decomp. (DSC) onset: 142.7 °C, peak max: 150.5 °C; R_f 0.36 (DCM/Et_2O, 70:30); (found: C, 56.35; H, 4.38; N, 11.50. $C_{17}H_{16}ClN_3S_2$ requires C, 56.42; H, 4.46; N, 11.61%); λ_{max}(DCM)/nm 242 inf (log ε 4.15), 301 (3.49), 388 (3.85), 410 inf (3.65); ν_{max}/cm^{-1} 3026w (aryl C-H), 2920w, 2837w and 2785m (alkyl C-H), 1582m, 1533w, 1497m, 1452m, 1410w, 1366m, 1342w, 1308w, 1292w, 1265w, 1236m, 1219m, 1171m, 1134m, 1099m, 1024m, 1013m, 901s, 860s, 799w, 764s; δ_H(500 MHz, acetone-d_6) 7.56 (2H, d, *J* 7.7), 7.43 (2H, d, *J* 7.2), 7.34 (2H, dd, *J* 7.5, 7.5), 7.30–7.20 (3H, m), 3.61 (4H, br s), 2.19 (3H, s); δ_C(125 MHz, $CDCl_3$) 157.8 (s), 149.6 (s), 148.1 (s), 138.8 (s), 137.8 (s), 130.3 (d), 129.0 (d), 128.3 (d), 127.1 (d), 119.5 (d), 61.9 (t), 61.2 (t), 42.2 (q); *m/*z (MALDI-TOF) 362 (MH^+, 49%), 360 (M^+-1, 100), 267 (4), 243 (28), 241 (65), 91 (3).

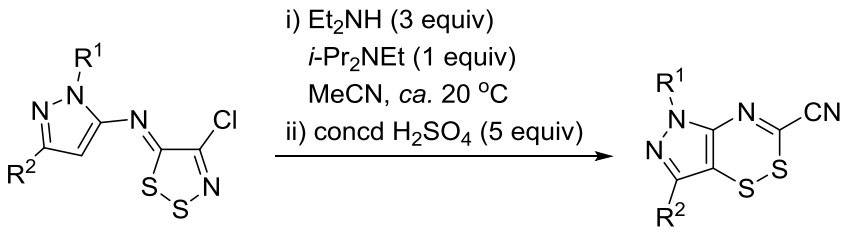
For the preparation of (6*H*-1,2,3-benzodithiazol-6-ylidene)propanedinitriles, **54** and **55**, substituted 6-chloro-1,2,3-benzodithiazol-2-ium chlorides **II** (Herz salts) were condensed with malononitrile (1

equiv) in the presence of base (2 equiv) according to the following general procedure: To a stirred suspension of the appropriate 6-chloro-1,2,3-benzodithiazol-2-ium chloride **II** (1 mmol) in dichloromethane (25 mL) at *ca.* 20 °C, malononitrile (66 mg, 1 mmol) was added followed by the addition of Hünig's base (348 μL, 2 mmol). After 1 h the reaction mixture adsorbed onto silica and dry flash chromatography gave the corresponding (6*H*-1,2,3-benzodithiazol-6-ylidene)propane-dinitriles **54** or **55**. Analytical and spectroscopic data of the (6*H*-1,2,3-benzodithiazol-6-ylidene)propanedinitriles **54** and **55** have been previously reported [20].



*2.2.3. Synthesis of 1,2,4-dithiazines **56-59***

The synthesis of 1,2,4-dithiazines **56-59** has been recently reported, [21] and involves the reaction of the appropriate (*Z*)-*N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-1*H*-pyrazol-5-amine **III** with diethylamine followed by treatment with concd H_2SO_4 according to the following general procedure: To a stirred suspension of the appropriate (*Z*)-*N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-1*H*-pyrazol-5-amine (0.2 mmol) in MeCN (4 mL) at *ca.* 20 °C was added Hünig's base (34.5 μL, 0.2 mmol) followed by diethylamine (63.0 μL, 0.6 mmol). After complete consumption of the starting material, to the mixture was added in one portion concd H_2SO_4 (55 μL, 1 mmol). The mixture was stirred for 5 min and then adsorbed onto silica and chromatographed to give the corresponding 5*H*-pyrazolo[3,4-*e*][1,2,4]dithiazine-3-carbonitrile. Analytical and spectroscopic data of the 1,2,4-dithiazines **56-59** have been previously reported [21].



III **56-59**

2.3. Purification of hLAT1

hLAT1 wild type and mutants C335A, C407A and C335A/C407A over-expressed in *E. Coli* were purified as previously described [3]. After cell lysate solubilisation and centrifugation (12,000 g, 10 min, 4 °C), the purification was performed using ÄKTA start. In particular, the supernatant was applied on a His Trap HP column (5 ml Ni Sepharose) equilibrated with 10 mL buffer (20 mM Tris HCl pH 8.0, 10% glycerol, 200 mM NaCl, 0.1% sarkosyl, and DTE 2 mM) while the protein was eluted with the same buffer plus 400 mM imidazole. Desalt of 2.5 mL of the purified protein was than performed using a PD-10 column.

2.4. Reconstitution of the purified hLAT1

The purified hLAT1 was reconstituted by removing detergent from mixed micelles containing detergent, protein and phospholipids using batch wise method as previously described [3]. The initial mixture contained: 4 μg of purified protein, 100 μL of 10% $C_{12}E_8$, 100 μL of 10% egg yolk phospholipids (*w/v*) in the form of liposomes prepared as previously described [22], 20 mM Tris HCl pH 7.5 and 10 mM L-His in a final volume of 700 μL.

2.5. Transport measurements

To remove the external substrate, 600 μL of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) pre-equilibrated with 20 mM Tris HCl pH 7.5 and sucrose at an appropriate concentration to balance internal osmolarity. Transport was initiated by adding 5 μM [^{3}H]His to proteoliposomes and stopped by a mixture of 100 μM BCH and 1.5 μM

$HgCl_2$. In controls, the mixture of inhibitors was added at time zero according to the inhibitor stop method [23]. 100 μL of proteoliposomes were passed through a Sephadex G-75 column (0.6 cm diameter × 8 cm height) at the end of the transport assay, to separate the external from the internal radioactivity. Proteoliposomes were eluted with 1 mL 50 mM NaCl in 4 mL scintillation mixture and counted. For [^{3}H]His uptake analysis, experimental values were corrected by subtracting controls. Initial transport rate was measured by stopping the reaction after 15 min for wild type protein and after 30 min for Cys mutants, *i.e.*, within the initial linear range of [^{3}H]His uptake in proteoliposomes. Grafit 5.0.13 software was used to calculate kinetic parameters and derive IC_{50} values in inhibition assays. Protein concentration was estimated by Chemidoc imaging system to calculate the hLAT1 specific activity [24].

2.6. Cell culture

HEK293 and SiHa cells, at late passage and kindly provided by Dr. Massimo Tommasino Infection and Cancer Biology group (IARC/CIRC- WHO/OMS), were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (*v/v*) fetal bovine serum (FBS), 1 mM glutamine and 1 mM sodium pyruvate and Pen/Strep as antibiotics. Cells were grown on 10 cm^2 plates at 37 °C in a humidified incubator and a 5% CO_2 atmosphere.

2.7. Transport measurement in HEK293 cells

pCDNA3hLAT1-HA was obtained by subcloning the cDNA of hLAT1 between BamHIII and XhoI restriction site into pCDNA3 mammalian expression vector. The HA tag was added by two sequential PCR reaction using the reverse 1 ATCTGGAACATCGTATGGGTATGTCTCCTGGGGGACCAC, then the reverse 2 CGGTCTCGAGCTAAGCGTAATCTGGAACATCGTATGGGTA and a single forward CGCGGATCCACCATGGCGGGTGCGGGCCCGAAG. The construct was verified by DNA sequencing. HEK293 cells were seeded onto 10 cm^2 plates and cultured using standard culturing conditions until they reached 80% confluence. Cells were transfected with Polyjet (Signagen

Laboratories) transfection reagent according to the manufacturer's procedures with 5 µg of pCDNA3hLAT1-HA or empty vector. After 24 h, empty vector or hLAT1 expressing cells were seeded onto 12 well plates and used for transport assay of L-[^{3}H]His. Cells were rinsed twice with warm transport buffer: 20 mM Tris HCl pH 7.4 and 5 mM glucose. Radiolabeled 5 µM [^{3}H]His was added and the transport reaction was terminated after 1 min by discarding the uptake buffer and rinsing the cells three times with the same ice-cold transport buffer (0.5 mL per well per rinse) plus 10 mM BCH. Cells from each well were solubilized in 500 µL of 1% TX-100 solution. Cell extracts were counted for radioactivity (400 µL). The remaining 100 µL in each well were used for protein concentration assay. Specific His-transport was evaluated by subtracting the transport values of cells transfected with hLAT1 to those transfected with empty vector. To verify hLAT1 over-expression, western blot analysis was conducted on cell lysates harboring or not hLAT1-HA. The protein was immuno-detected incubating membrane with anti-HA (Sigma Aldrich) antibody 1:1000 overnight at 4 °C. As loading control 1:5000 anti-Actin (Sigma Aldrich) was used 1 h at room temperature. The reactions were detected by Electro Chemi Luminescence (ECL) assay after 1 h incubation at room temperature with secondary antibody anti-mouse (Cell Signaling) 1:5000.

2.8. Cell viability

SiHa cells were seeded onto 6 well plates and cultured using standard culturing conditions until they reached 70% confluence. Cells were treated with 10 and 100 µM of dithiazole inhibitors as indicated in the figure legends. Cell viability were followed for 24 and 48 h and pictures were taken.

2.9. Computational studies

Outward-open hLAT1 structure was obtained by comparative modeling, using the AdiC crystallographic structure (RCSB PDB code: 3OB6) as a template [25]

Tested compounds were drawn and energy minimized by the Molecular Operating Environment (MOE) Builder, with the Amber10:EHT force field. Since 1,2,3-dithiazoles can react with

nucleophile compounds, such as C335 and C407, the MOE Dock program was used with the covalent feature.

The top-scoring poses from the docking procedure were refined by using the MOE QuickPrep procedure aimed at refining the complex before calculating the approximate binding free energy via the GBVI/WSA dG empirical scoring function [26].

2.10. Statistical analysis

Data points of each experiments derived from samples in triplicates. Threshold for statistical significance (P value) was fixed at 0.01 according to Student's two tailed unpaired T-test. Data reported in Table 1 have been obtained from two experiments for a preliminary screening of the compounds.

3. Results

*3.1. Inhibition of the hLAT1 transporter by 1,2,3-dithiazoles **1-55** and 1,2,4-dithiazines **56-59***

Title compounds **1-59** (Table 1) were prepared and tested as inhibitors of hLAT1. Transport activity was assayed in proteoliposomes as [^{3}H]His$_{ex}$/His$_{in}$ antiport. Results of this preliminary screening, performed at 100 µM of each compound, are reported in Table 1 as percent of inhibition. The concentration used was about three times the threshold fixed for significant xenobiotic/protein interactions [27]. Several compounds triggered virtually complete inhibition, *i.e.* >90% (Table 1, compounds **5**, **10**, **11**, **12**, **16**, **17**, **19** and **59**); the other tested molecules showed a variable extent of inhibition ranging from 0 to 89%. No effect was exerted by DMSO used to solubilize the compounds, demonstrating that the inhibition could be ascribed exclusively to the compounds.

3.2. Dose-response analysis

The compounds showing inhibition higher than 90% at 100 µM were selected for dose-response analyses.

In these experiments, the rate of the [^{3}H]His/His antiport was determined in the presence of increasing concentrations of the inhibitors (Fig. 1). As expected, all the selected compounds led to a strong and complete inhibition with IC_{50} values of: 0.98 ± 0.10, 1.33 ± 0.33, 1.62 ± 0.43, 2.10 ± 0.58, 1.62 ± 0.30, 0.89 ± 0.33, 1.87 ± 0.09 and 5.2 ± 0.11 μM for compounds **5**, **10**, **11**, **12**, **16**, **17**, **19** and **59**, respectively (Fig. 1).

3.3. Molecular mechanism of interaction/inhibition

Dithiazoles and dithiazines are expected to interact with the protein through formation of mixed disulfide/trisulfide as previously reported ([15, 28, 29] and see Fig. 2). Human LAT1 harbours 12 Cys residues in its structure; thus, a strategy for assessing the possible reaction with thiol residues was adopted (Fig. 3): proteoliposomes were treated or not with the two most potent inhibitors, *i.e.* compounds **5** and **17** of Table 1. Treatment was followed or not by incubation with the disulfide reducing agent DTE. After incubation, excess of unreacted compounds was removed by a second size-exclusion chromatography. Then, His transport was assayed as described above. By adopting this strategy, the formation of covalent bonds between the inhibitor and the transporter were assessed as well as the recovery of transport function by DTE (Fig. 4). The concentration of compounds was chosen little higher than the IC_{50} to carry out experiments under strong inhibiting conditions. As shown in Fig. 4, compounds **5** (Fig. 4 A) and **17** (Fig. 4 B) led to inhibition of about 75% which was stable, *i.e.*, still present after removal of the compounds by size exclusion chromatography (Fig. 3). That inhibition was caused by formation of mixed di(tri)sulfides (Fig. 2) was confirmed by the action of DTE that induced a consistent recovery of LAT1 transport activity (Fig. 4).

3.4. Kinetics of inhibition

To gain further insights into the molecular mechanism of action, inhibition kinetics was investigated. Experiments were conducted by studying the dependence of transport rate on His concentration in the presence of the compounds **5** and **17** used at concentrations close to their IC_{50}

(Fig. 5). The data were analysed by double reciprocal plots, according to Lineweaver-Burk, from which straight line patterns intersecting on or close to the *x*-axis was found. Mixed or non-competitive inhibition was found for compound **5** (Fig. 5 A). Mixed inhibition was found for compound **17** (Fig. 5 B). The half saturation constant K_i, calculated from the experiments were 0.76 ± 0.27 μM and 1.13 ± 0.41 μM for compounds **5** and **17**, respectively (Fig. 5 A-B). The potential protection of inhibition by substrate was also tested. Pre-incubation of proteoliposomes with His at concentrations up to 10 mM was performed prior to dithiazoles. No protection by His was observed with compound **5** (Fig. 5 C), in agreement with the covalent mechanism, *i.e.* non-competitive, of the inhibition described.

3.5. Homology model and effects on LAT1 mutants

The reactivity of dithiazoles towards SH groups of the protein allowed to hypothesize the involvement of one or more Cys residues of the protein as the nucleophilic group attacking the cyclic disulfide bond on the inhibitor. The availability of 3D homology model [3] allowed us to perform covalent docking simulations both on C335 and C407 for compounds **5** and **17**, showing that both these compounds can bind C407 (covalent docking score and approximate binding free energy for compound **5**: -4.8 kcal/mol and -21.4 kcal/mol, respectively; covalent docking score and approximate binding free energy for compound **17**: -3.1 kcal/mol and -14.6 kcal/mol, respectively) (Fig. 6), whereas both the tested compounds cannot covalently bind C335; moreover, compound **51** has been used as negative control of docking (covalent docking score for compound **52**, 125.6 kcal/mol) being a poor inhibitor of LAT1 (Table 1).

Interestingly, the identified residue was C407 located in the substrate binding site as previously demonstrated [3]. Thus, the C407A mutant was used for dose-response analyses performed on compound **5**. This was chosen due to its higher affinity (K_i value), confirmed by the good covalent docking score and approximate binding free energy obtained in silico. The calculated IC_{50} of the C407 mutant was 3.76 ± 0.28 μM, *i.e.* four times higher than that of wild type (Fig. 7A). The

substrate binding site includes also C335 for which docking analysis predicted no interaction with both compounds **5** and **17**. Indeed, IC_{50} of C335A mutant derived from dose-response analysis on compound **5** was 1.32 ± 0.16 µM (Fig. 7B), *i.e.* similar to that of wild type (Fig. 1A). To further ascertain the exclusive role of C407, the IC_{50} was calculated also for the double mutant C335A/C407A (Fig. 7C). In this case, a value of 4.60 ± 0.39 µM was obtained, similar to that of the single mutant C407A.

3.6. Effects on intact cells

Given the strong inhibitory effect of compound **5**, inhibition experiments were performed on intact HEK293 cells transiently transfected with hLAT1-HA. Fig. 8A demonstrates the actual over expression of hLAT1 detected by an anti-HA antibody as control. [^{3}H]His uptake was measured in the presence or absence of compound **5** (Fig. 8B) at a concentration corresponding to its IC_{50} in proteoliposomes (Fig. 1A). Transport in HEK293 cells was inhibited by about 50% confirming that the compound inhibits hLAT1 in intact cells at the same extent as in proteoliposomes. To gain further insight on the biological effects of inhibitors, cell viability was tested. In this case, SiHa cell line was used since these cells harbor a detectable level of endogenous LAT1 [30]. The experiment was conducted by monitoring cell morphology 24 and 48 h after addition of increasing concentrations of the most active compounds **5** and **17** or the negative control compound **51** that showed <37% inhibition in proteoliposomes (Table 1); DMSO was added to control cells, *i.e.*, in the absence of inhibitor (Fig. 9). Interestingly, the compounds with the lowest IC_{50} were very effective in inducing apparent cell death triggering significant reduction of cell numbers together with evident changes in cell morphology. The effects were more pronounced after 48 h. Interestingly, after washing out the compounds, cell viability was still impaired. Compound **51** was used as specificity control being one of the compounds with the lowest inhibitory effects on LAT1. Notably, the compound did not cause cell death highlighting the specificity of the tested dithiazoles with respect to hLAT1 activity.

4. Discussion

In the last 10 years, more than 200 papers appeared on Pubmed database ["LAT1" (or aliases) and "cancer" (or aliases) as query in Title/Abstract] dealing with LAT1 over-expression in several human cancers. On this basis, the transporter became acknowledged as a new pharmacological target for cancer therapy. LAT1 has been also exploited for delivery of pharmacological compounds which mimic its substrates as proposed in the pro-drug approach [31]. Proteoliposomes reconstituted with hLAT1 have been used in the present work as the main experimental model for screening of inhibitors due to its intrinsic simplicity being not affected by other transport activities. Data on inhibitors have been then confirmed on experimental models closer to the *in vivo* conditions.

The task of designing good ligands/inhibitors for LAT1 is challenging, as for most membrane transporters, owing to the absence of its 3D crystallographic structure. Indeed, LAT1 structures were derived, so far, by homology modeling on the basis of the arginine/agmatine transporter, *i.e.* AdiC from *E. coli* [3, 25, 32-34], sharing a relatively low sequence identity with hLAT1. From these models, some features of LAT1 substrate binding site were predicted and exploited for designing not transported substrate-mimicking molecules, with the aim of finding potent competitive inhibitors [4, 35-38], following a strategy firstly used for designing inhibitors of the ASCT2 amino acid transporter [39]. A limitation of this type of inhibitors resides in the competition exerted by endogenous amino acid substrates which have plasma concentrations higher than the K_m for transporters [10, 40]. In this case, inhibitors can be displaced from the transporter site leading to only transient or inefficient effects [35]. Herein, an alternative strategy has been attempted taking into account the information recently obtained on structure-function relationships of LAT1 substrate site [3]. Indeed, we exploited the presence of Cys residues in the substrate binding site of the protein to obtain, on the one hand, a more potent inhibition in terms of affinity, on the other hand, an irreversible inhibition mechanism to chemically knocking-out the transporter. Thus, a number of

electrophilic molecules has been tested, likely reacting covalently with the thiolate group of a Cys, that are dithiazoles and dithiazines, known for their antibacterial, antifungal, herbicidal and anticancer effects [41-49]. Noteworthy, some of these compounds were previously described as covalent inhibitors of rat ASCT2 transporter [15].

The variety of chemical substituents on the 1,2,3-dithiazole and to a lower extent on the 1,2,4-dithiazine rings allowed us to discover a number of compounds with a very high affinity for LAT1 showing the lowest IC_{50} achieved so far, *i.e.*, half the previous lowest value [36]. Regarding reactivity of the compounds, the S2 atom of the disulfide motif (Fig. 2), would represent the preferred electrophilic site for the attack by a thiolate of a Cys residue of the protein but the nucleophilic attack on S1 cannot be excluded. The reaction mechanism may therefore occur via the formation of mixed di- or trisulfides, as previously suggested for the reaction of the same compounds with another amino acid transporter [15]. The mixed disulfide mechanism has been adopted (Fig. 2) for docking calculations since this is the most widely occurring mechanism in vivo. That inhibition was due to covalent interaction was demonstrated herein for the most effective compounds **5** and **17**. Three different strategies were adopted to identify the mechanism of inhibition and the Cys residue targeted by these compounds. Firstly, the significant activity recovery induced by the thiol reducing agent DTE indicated involvement of Cys residue(s) in the covalent interaction with compounds. However, in the case of compound **17**, DTE did not allow 100% recovery of LAT1 transport activity probably because of additional hydrophobic interactions independent from thiol reactivity. Secondly, the kinetics showed a non-competitive and mixed type inhibition for both compounds **5** and **17** that supported the covalent interaction with LAT1. In line with these experimental data, C407 was definitively identified as the target of compound **5** by site-directed mutagenesis.

C407, being located in the substrate binding site may be easily blocked by mixed sulfide formation triggering the competitive component of the inhibition. Accordingly, the pre-incubation of

reconstituted proteoliposomes with excess of substrate did not alter the inhibition potency by compounds **5**.

Some information on SAR trends can be obtained from the IC_{50} of the eight most active compounds and from the % of inhibition by all the others. It seems likely that electron withdrawal substituents on the phenyl ring facilitate the interaction/binding to the protein resulting in a higher inhibitory effect, with the exception of compound **2**. In contrast, electron-donating and bulky substituents, along with polycyclic compounds, are in general less effective in blocking LAT1 transport activity. The pyrid-2-yl sp^2 nitrogen lone pair of dithiazoles **26-29**, **32** and **33** donates electron density into the antibonding S-S bond of the dithiazole and this raises the electron density of the ring, making it less prone to thiophilic attack. This explains the sudden drop in activity of such dithiazoles while, interestingly, the introduction of electron withdrawing groups (as the cyano group in **27**) restores some activity. This electronic effect was recently detected in the evaluation of 1,2,3-dithiazoles reactivity [29].

Interestingly, the strategy adopted in this work responded also to the 3R requirement for animal testing: Replace, Reduce, Refine. In fact, out of 59 compounds 8 hits were identified and, among them, 2 lead compounds were chosen for a deep investigation. Only at the very end of the screening, the selected compounds, for which the molecular mechanism of action was deciphered in proteoliposomes, were used to verify effects on cell viability. Taken together, the collected results defined, with relatively low cost and no animal experimentation, the molecular scaffold that could be used for designing potential drugs to be used in more advanced pre-clinical trials.

Conflicts of interest

The authors declared that they have no competing interests.

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Legends to Figures

Fig. 1. Dose-response curves for the inhibition of the recombinant hLAT1 in proteoliposomes. Transport was measured adding 5 μM [^{3}H]His to proteoliposomes containing 10 mM His in the presence of different concentrations of the indicated compounds and measured in 15 min as described in Materials and Methods. Dose-response for compound **5** (A), compound **10** (B), compound **11** (C), compound **12** (D), compound **16** (E), compound **17** (F), compound **19** (G) and

compound **59** (H). Percent residual activity with respect to the control (without additions) is reported. Results are mean ± S.D. from three independent experiments.

Fig. 2. Possible pathways for the mechanism of hLAT1 transporter inhibition by compounds. Cys-S, thiolate group of a Cys residue.

Fig. 3. Sketch of experimental design for assessing the possible reaction of the compound with thiol residues of the protein. After reconstitution, proteoliposomes were incubated with DMSO (control) or with a specific compound (Inhibition assay) for the indicated time. Successively these samples were incubated with buffer alone or with 50 mM DTE. Transport was then measured in 30 min by adding 5 μM [^{3}H]His to proteoliposomes, as described in Materials and Methods.

[1]: A first size exclusion chromatography was performed to remove the external substrate after reconstitution.

[2]: A second size exclusion chromatography was performed to remove inhibitor not bound to the transporter.

Fig. 4. Effect of DTE on the inhibition of [^{3}H]His uptake in proteoliposomes reconstituted with recombinant hLAT1. Experimental procedure is described in the legend of Fig. 3. Proteoliposomes were incubated with DMSO or with 1.2 μM compound **5** (A), 1.2 μM compound **17** (B), and not treated or treated with 50 mM DTE. Results are mean ± S.D. from three independent experiments. Student's two tailed unpaired t-test was performed on the sample without added compounds (control); p value was symbolized as follows: *$p < 0.01$.

Fig. 5. Kinetic analysis of the inhibition of recombinant hLAT1 reconstituted in proteoliposomes. Data were plotted according to Lineweaver–Burk as reciprocal transport rate vs reciprocal His concentration. Transport rate was measured by adding [^{3}H]His at the indicated concentrations to proteoliposomes containing 10 mM His and stopping the reaction after 15 min as described in Materials and Methods. In (A) 0.8 μM (●) or 1.2 μM (□) **5** was added as inhibitor in comparison to samples without inhibitor (○). In (B) 0.8 μM (●) or 1.2 μM (□) **17** was added as inhibitor in comparison to samples without inhibitor (○).Results are mean ± S.D. from three independent

experiments. (C) Protection of inhibition by substrate. Proteoliposomes reconstituted as described in Materials and Methods, were incubated or not with compound **5 i**n the presence or absence of **5** or 10 mM His. After incubation, samples were subjected to size exclusion chromatography to remove the unreacted inhibitor and substrate; transport rate was measured adding 5 μM [^{3}H]His. The reaction was stopped after 30 min as described in Materials and Methods. Percent residual activity with respect to the control (without additions) is reported. Results are mean ± S.D. from three independent experiments. Student's two tailed unpaired t-test was performed on the sample without added compounds (control); p value was symbolized in (C) as follows: *$p < 0.01$.

Fig. 6. Best docking poses of the two selected compounds obtained through covalent docking on LAT1 homology model. LAT1 in its outward-open conformation is shown as ribbon; the gate residue Phe252 is shown in stick representation. Compounds **5** and **17** covalently docked to residue Cys407 are shown in stick representation in A and B, respectively.

Fig. 7. Inhibition analysis of compound **5** on recombinant hLAT1 mutants reconstituted in proteoliposomes. Dose-response curve for the inhibition of compound **5** on LAT1 C407A (A), on C335A (B) and on double mutant C335A/C407A (C). Transport was measured by adding 5 μM [^{3}H]His to proteoliposomes containing 10 mM His in the presence of different concentrations of the compound added together with [^{3}H]His and measured in 30 min as described in Materials and Methods. Percent residual activity with respect to the control (without additions) is reported. Results are mean ± S.D. from three independent experiments.

Fig. 8. Effect of compound **5** on transport activity in intact cells. (A) Western Blot analysis was conducted using anti-HA antibody on cells transfected with empty vector (pCDNA3) or hLAT1-HA (pCDNA3-LAT1) as described in Materials and Methods. Actin is used as loading control. Picture is representative of three independent experiments. (B) HEK293 cells were cultured and transfected with pCDNA3-hLAT1-HA as described in Materials and Methods and used for transport adding 5 μM[^{3}His] and 1 μM of compound 5. Transport was stopped after 1 min as described in Materials and Methods. Results are mean ± S.D. from three independent experiments. Student's two tailed

unpaired t-test was performed on the sample without added compound (control); p value was symbolized as follows: *$p < 0.01$.

Fig. 9. Effect of compounds on cell viability. SiHa cells were cultured in 6 well plates as described in Materials and Methods. Compounds **5** (A), **17** (B) and **51** (C) were added at the indicated concentrations and pictures were taken after 24 h and 48 h of treatment. As negative control, DMSO was added. After 48h medium was replaced with fresh DMEM without inhibitors (wash) and pictures were taken after 24h. Picture is representative of three independent experiments.

Figure 1

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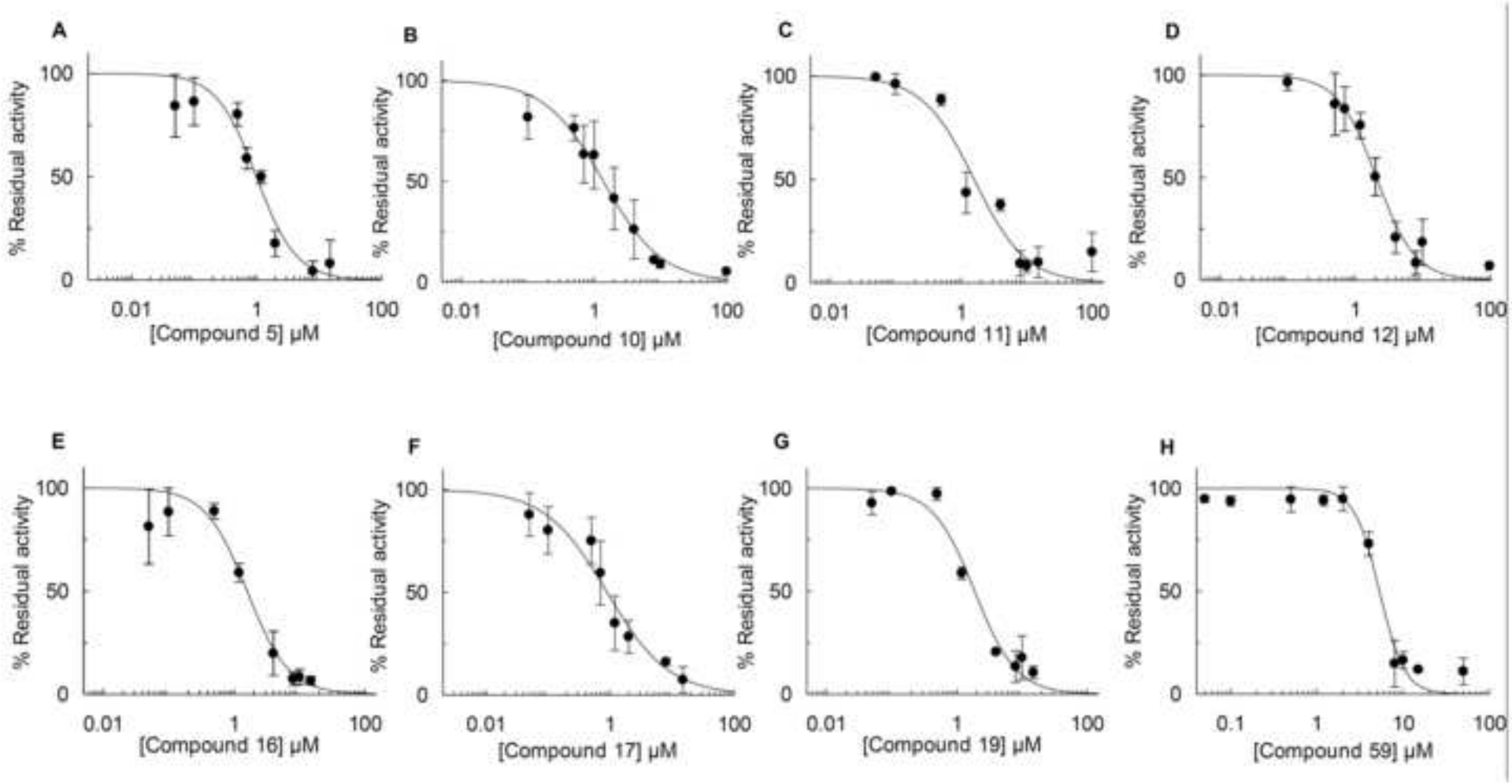

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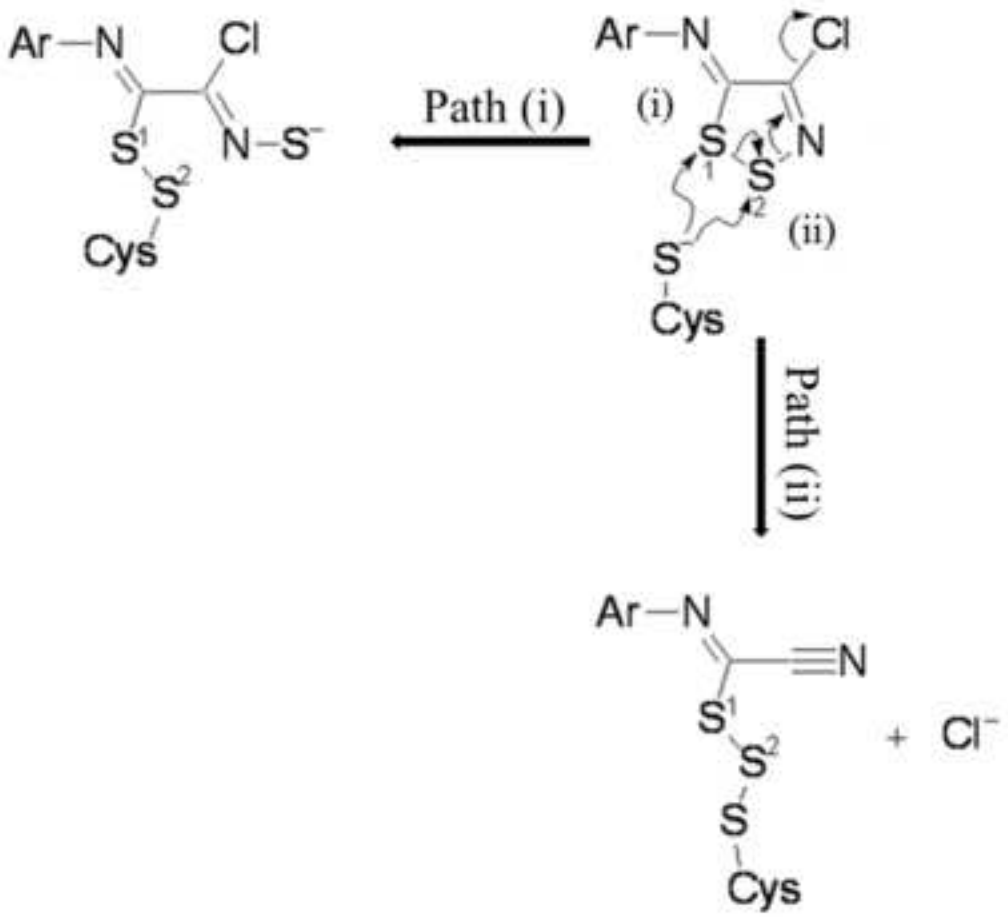

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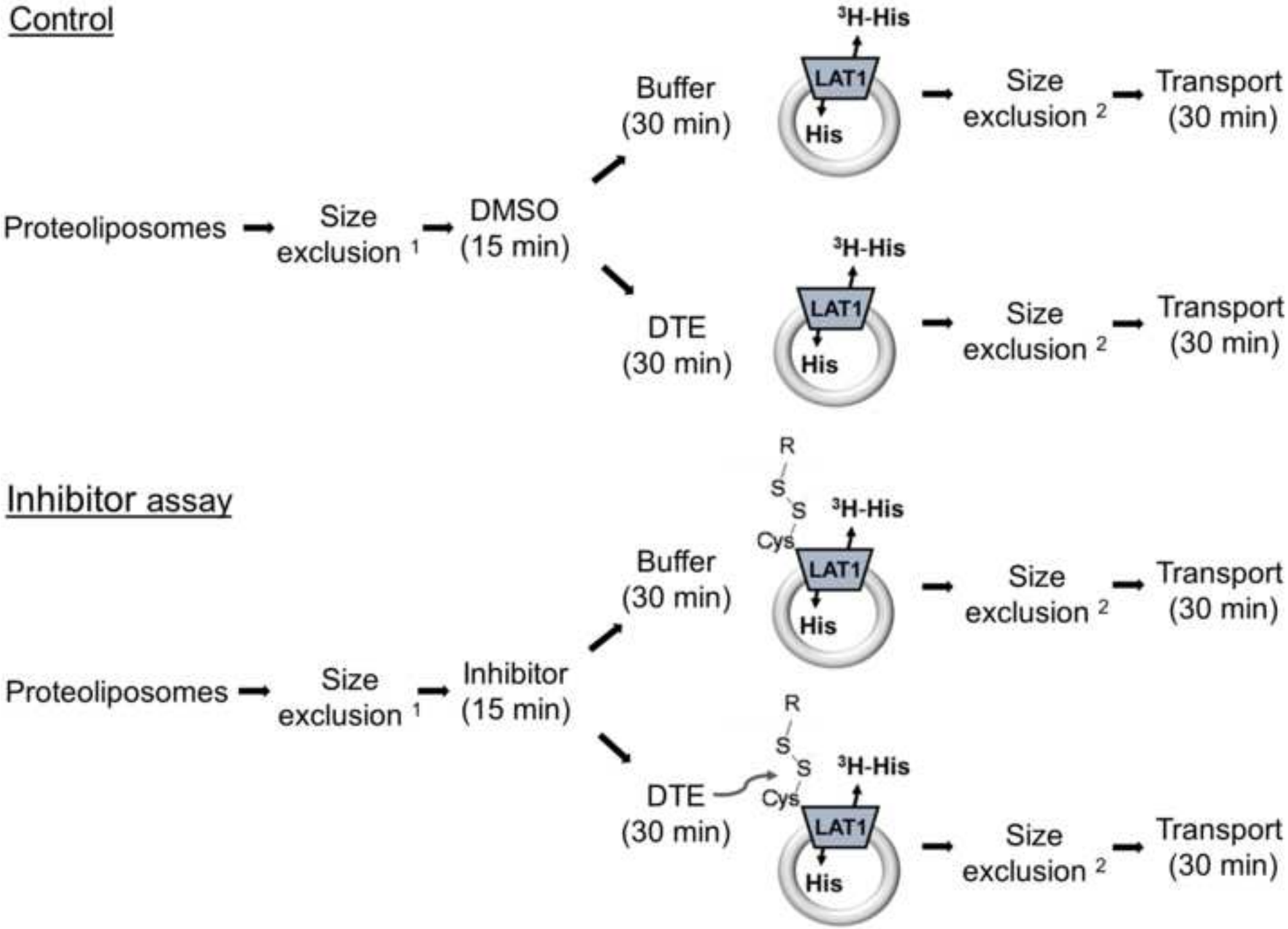

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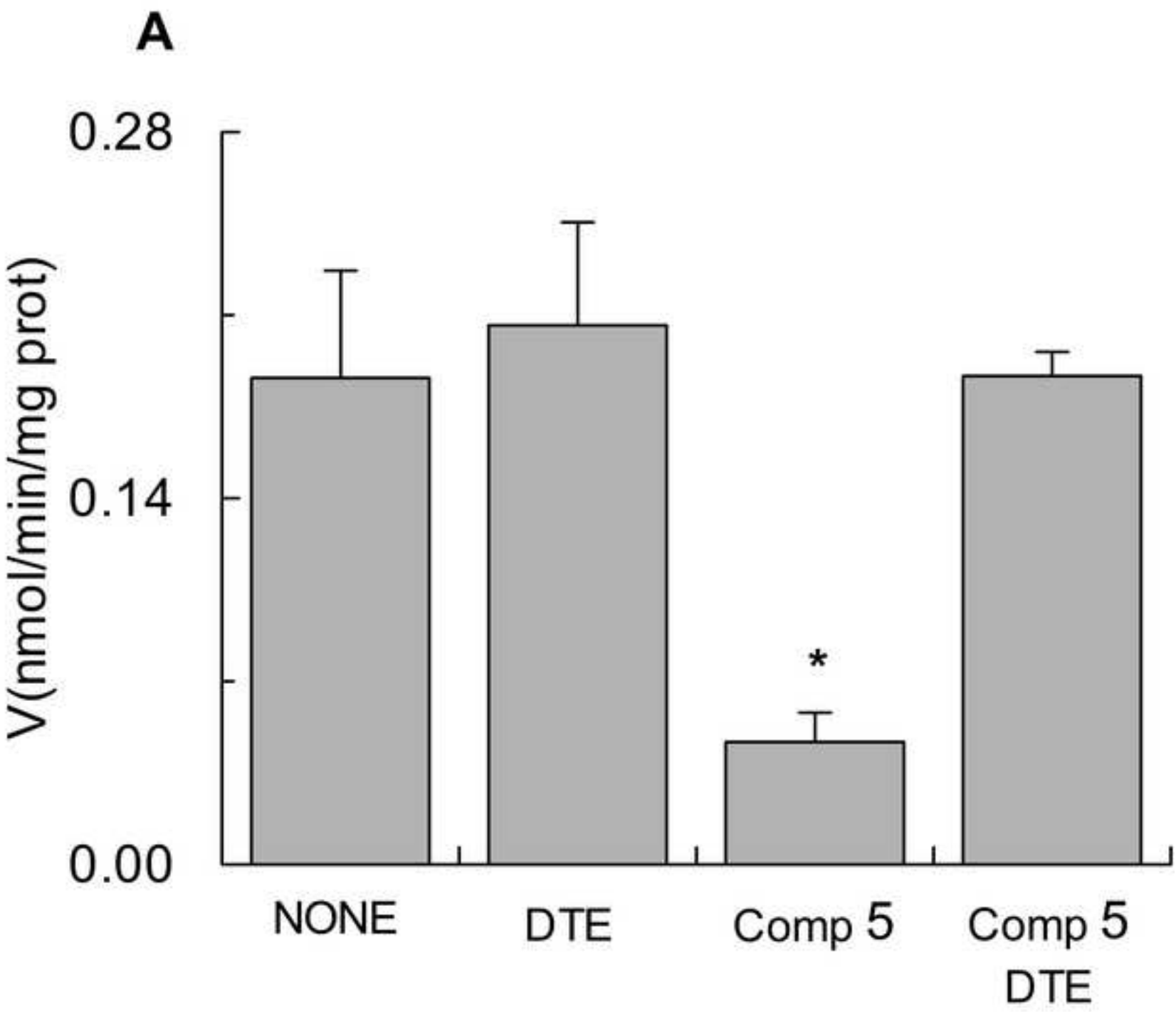

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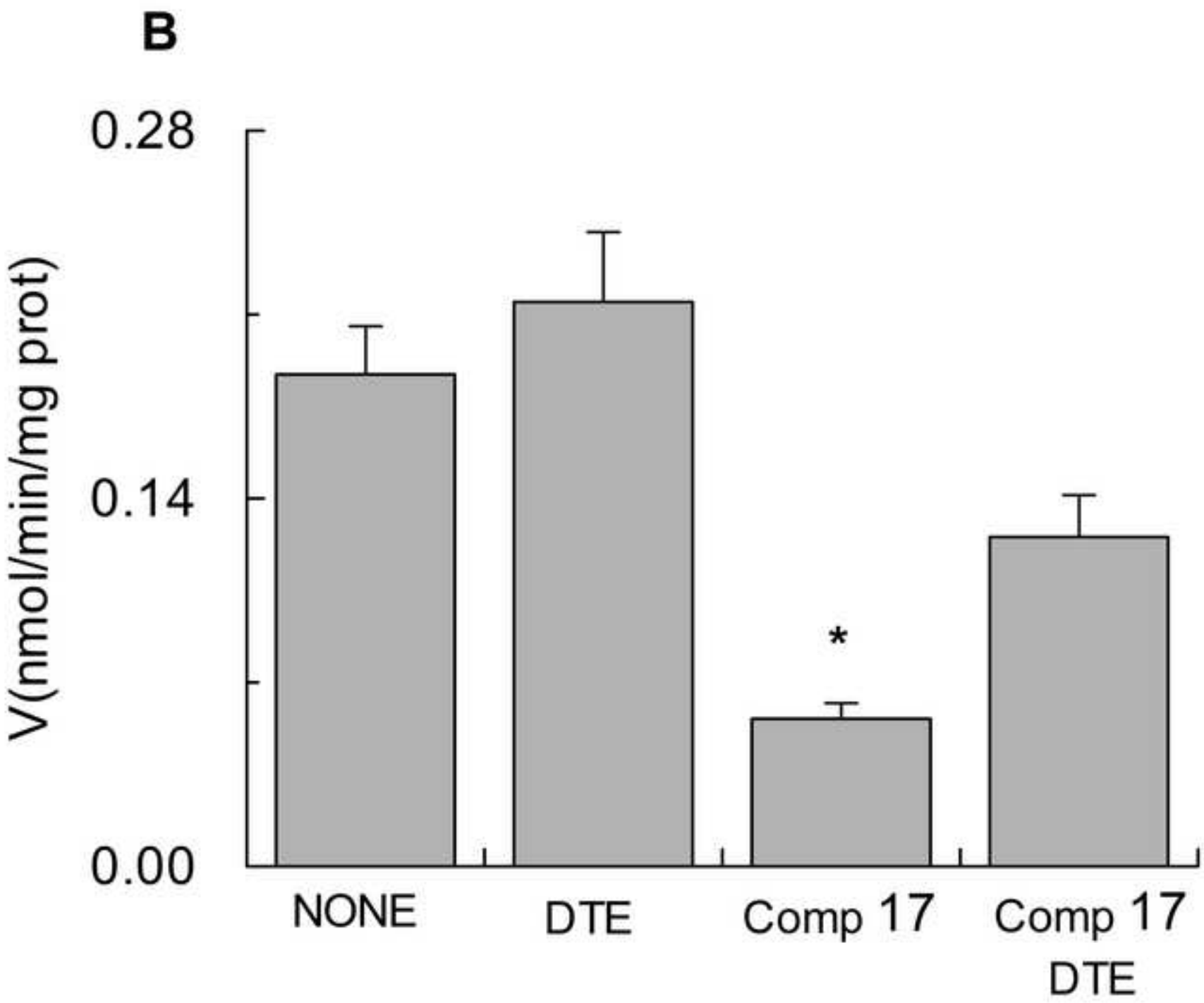

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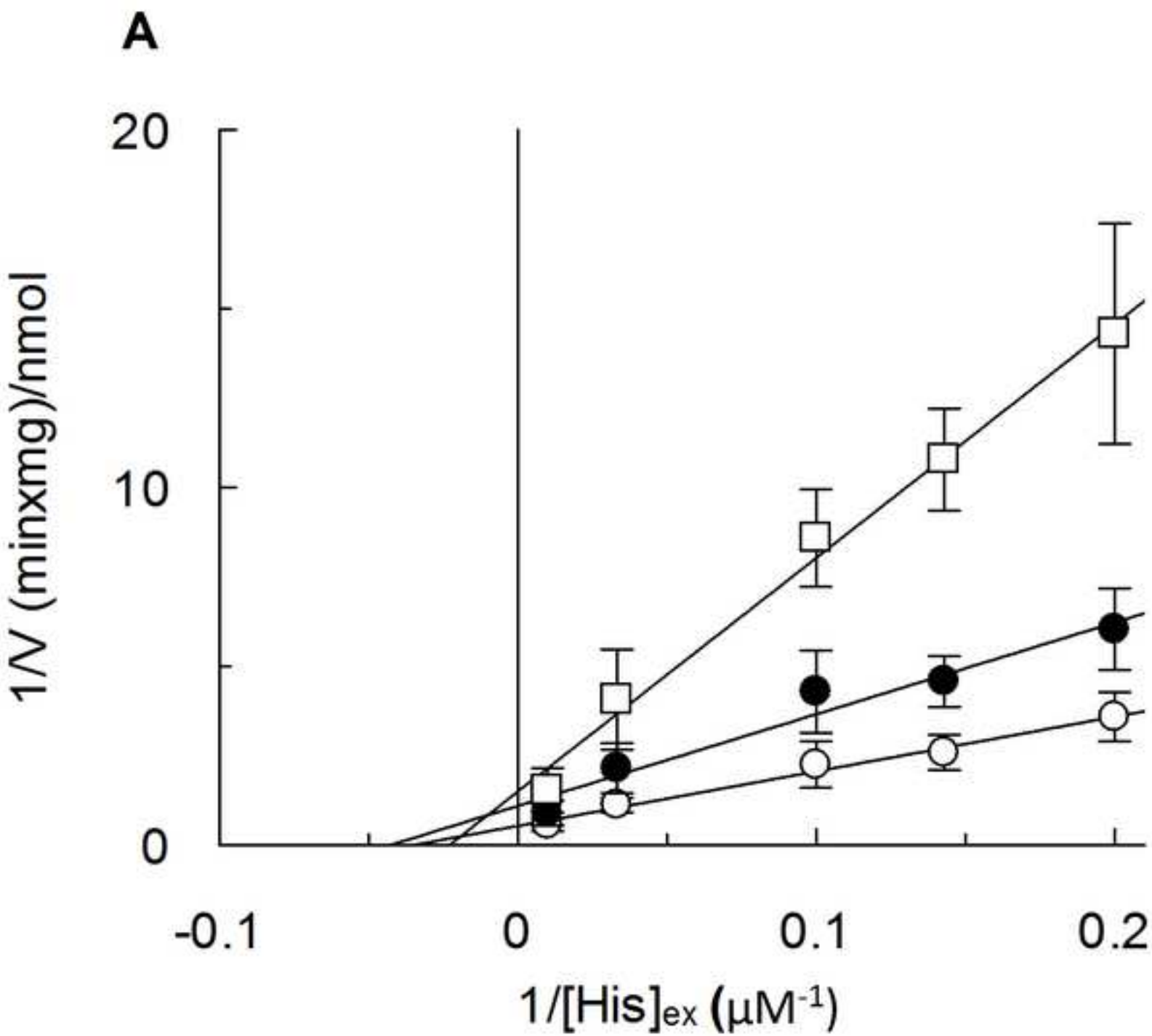

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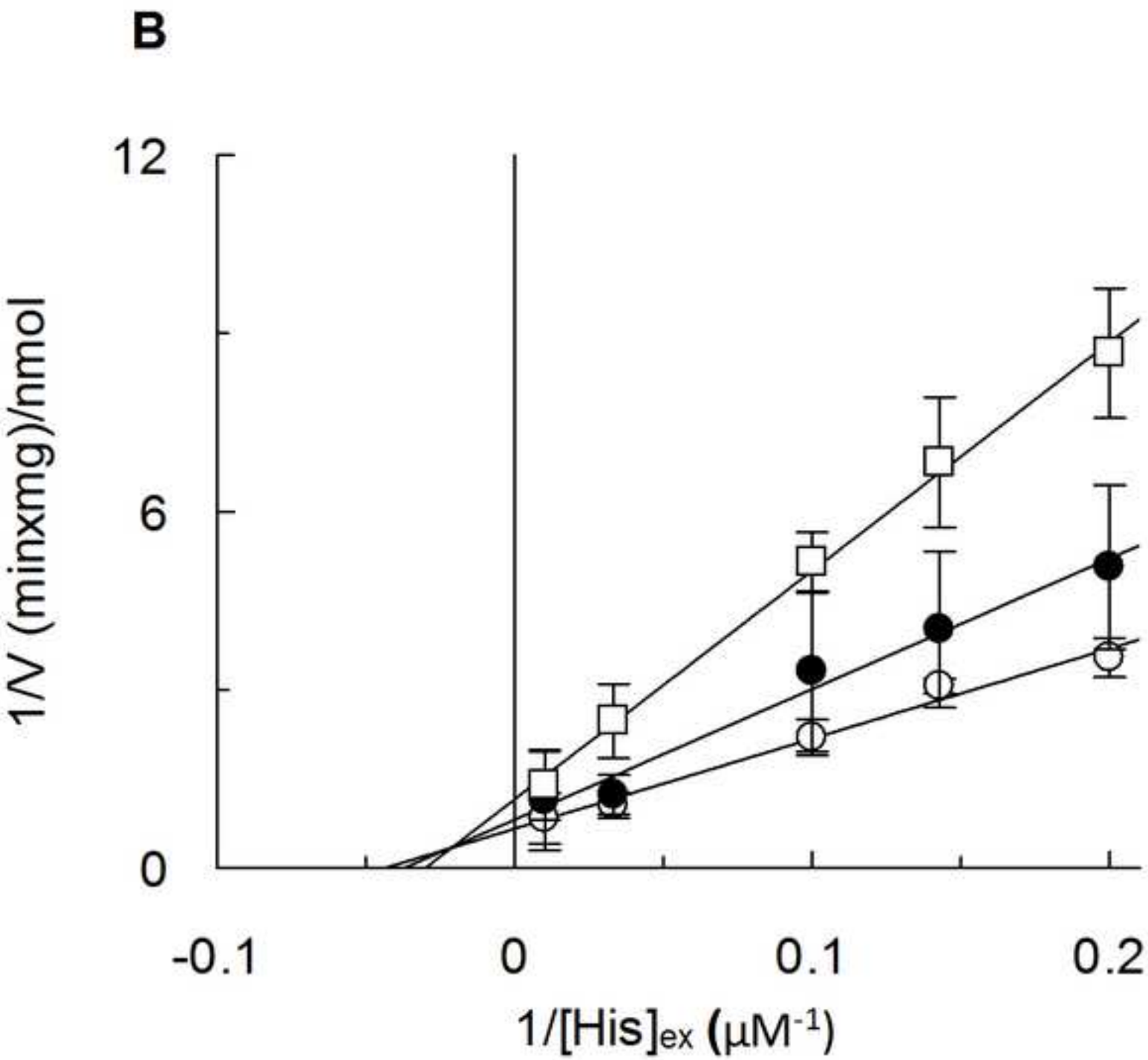

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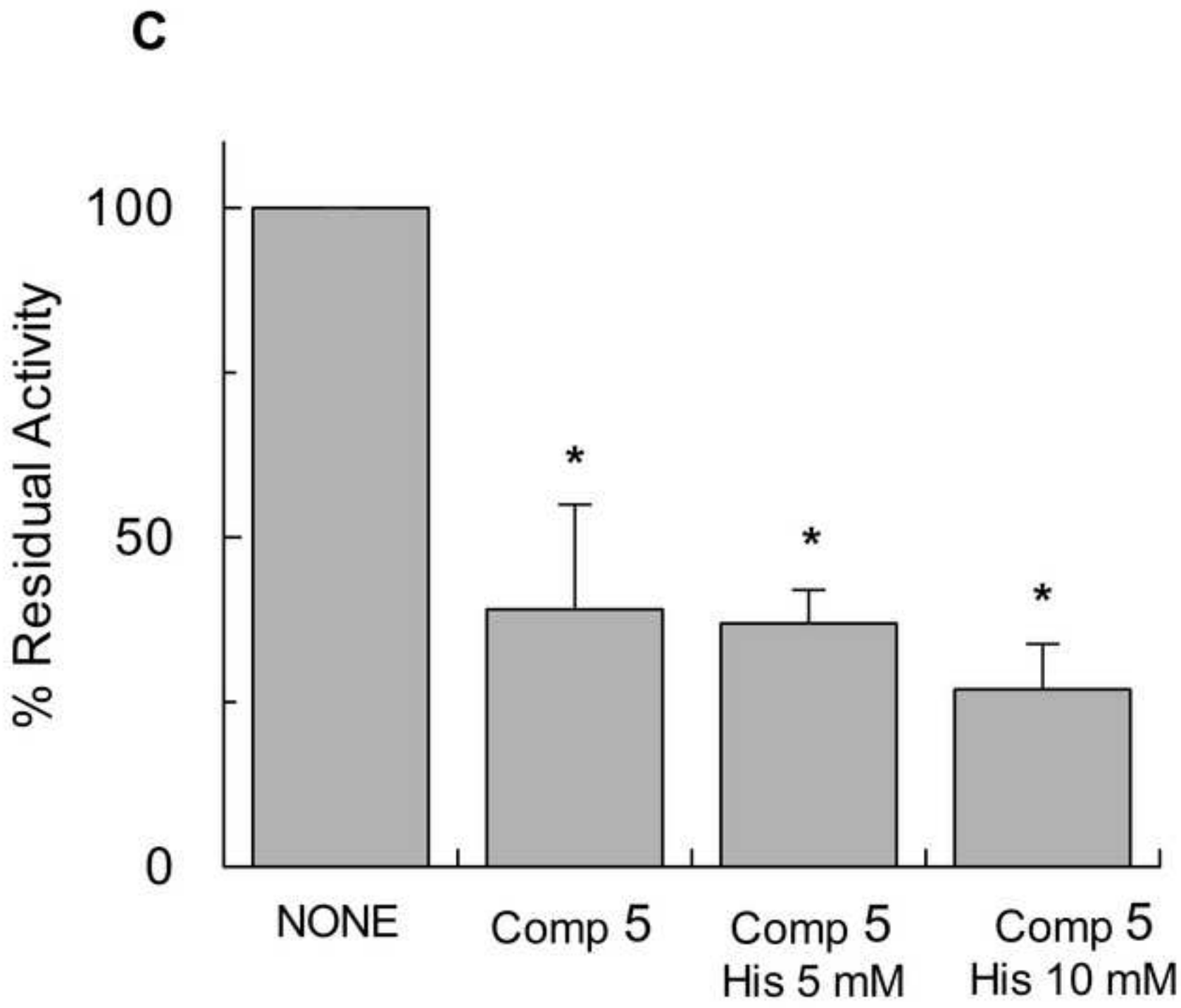

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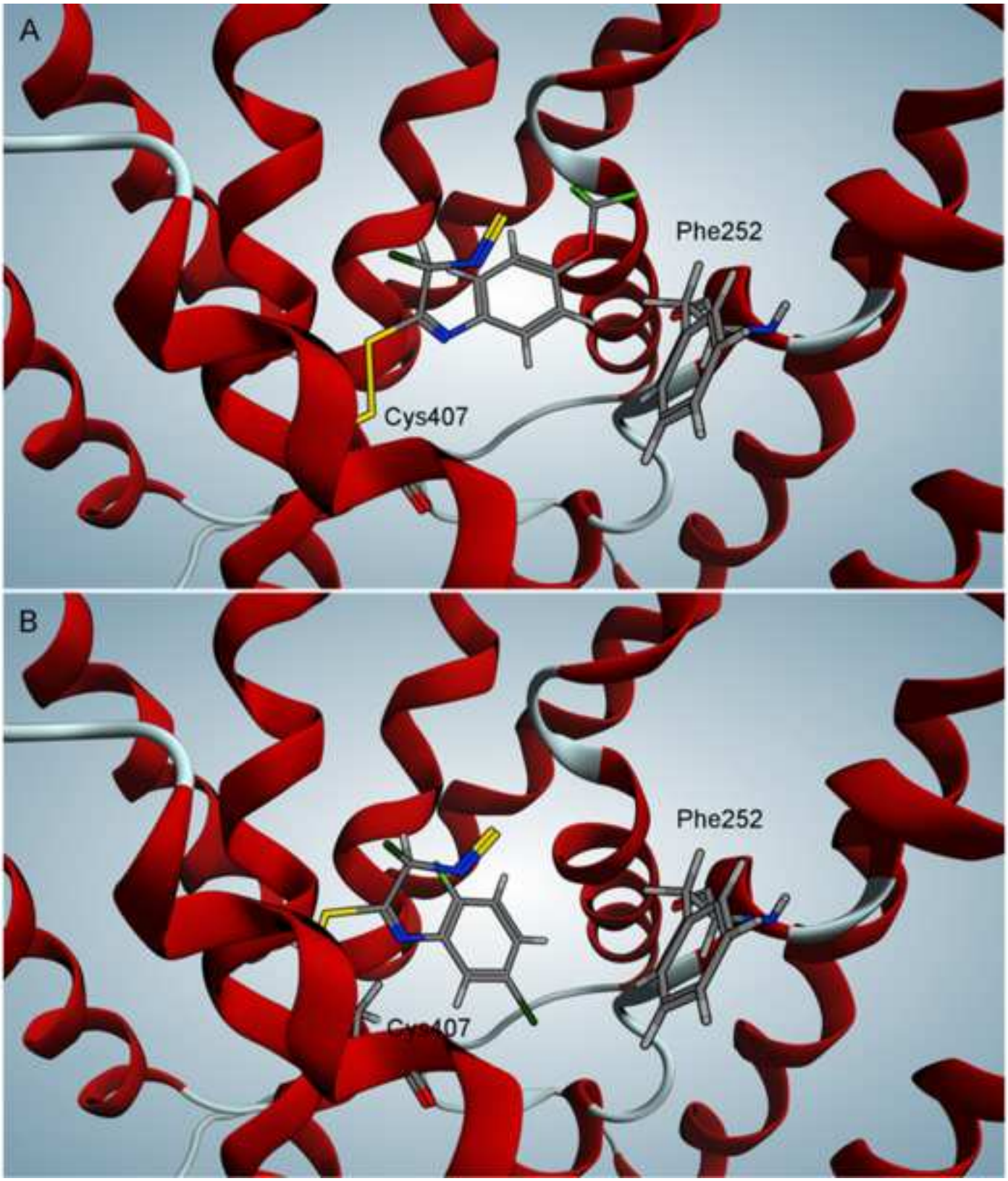

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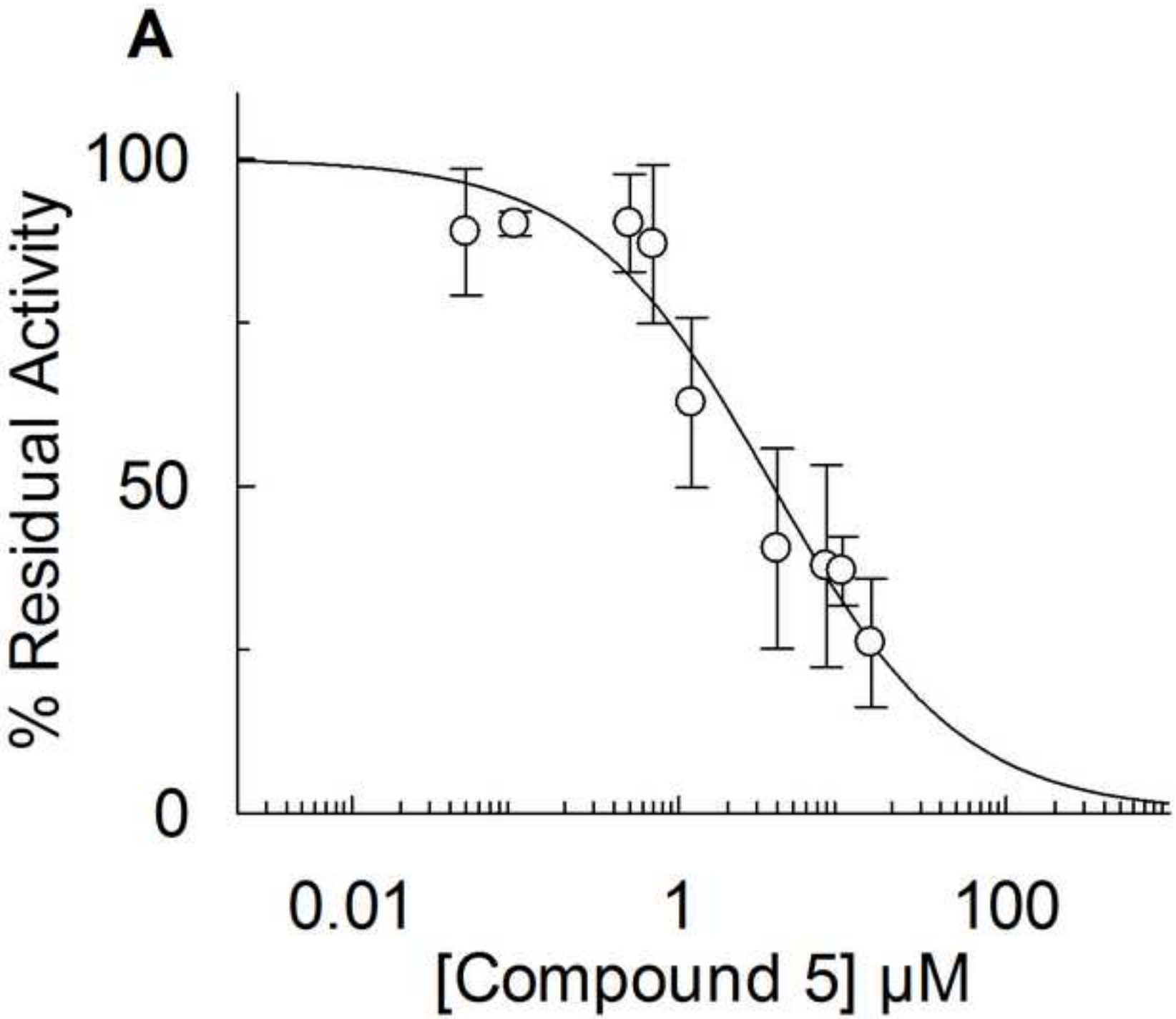

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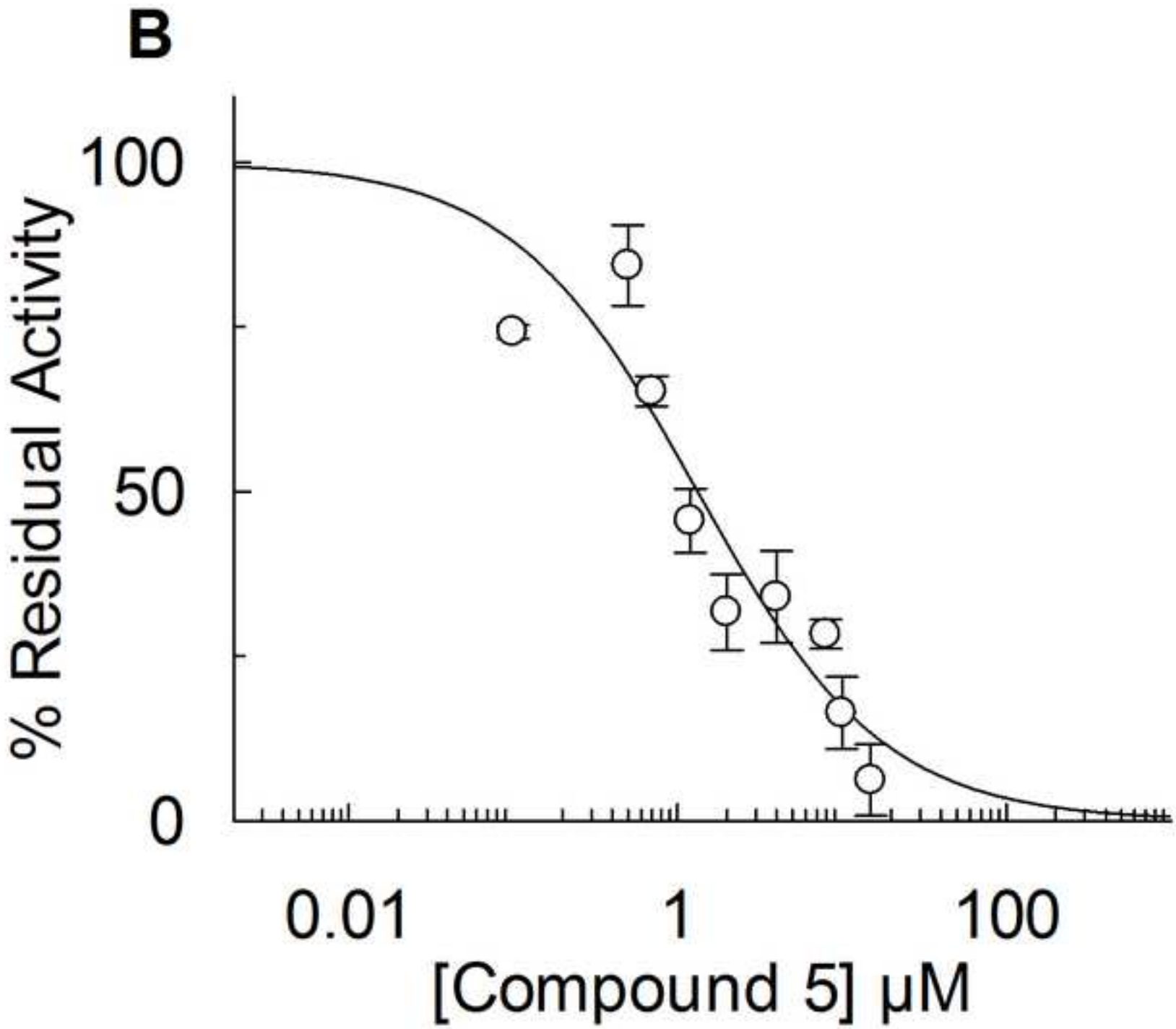

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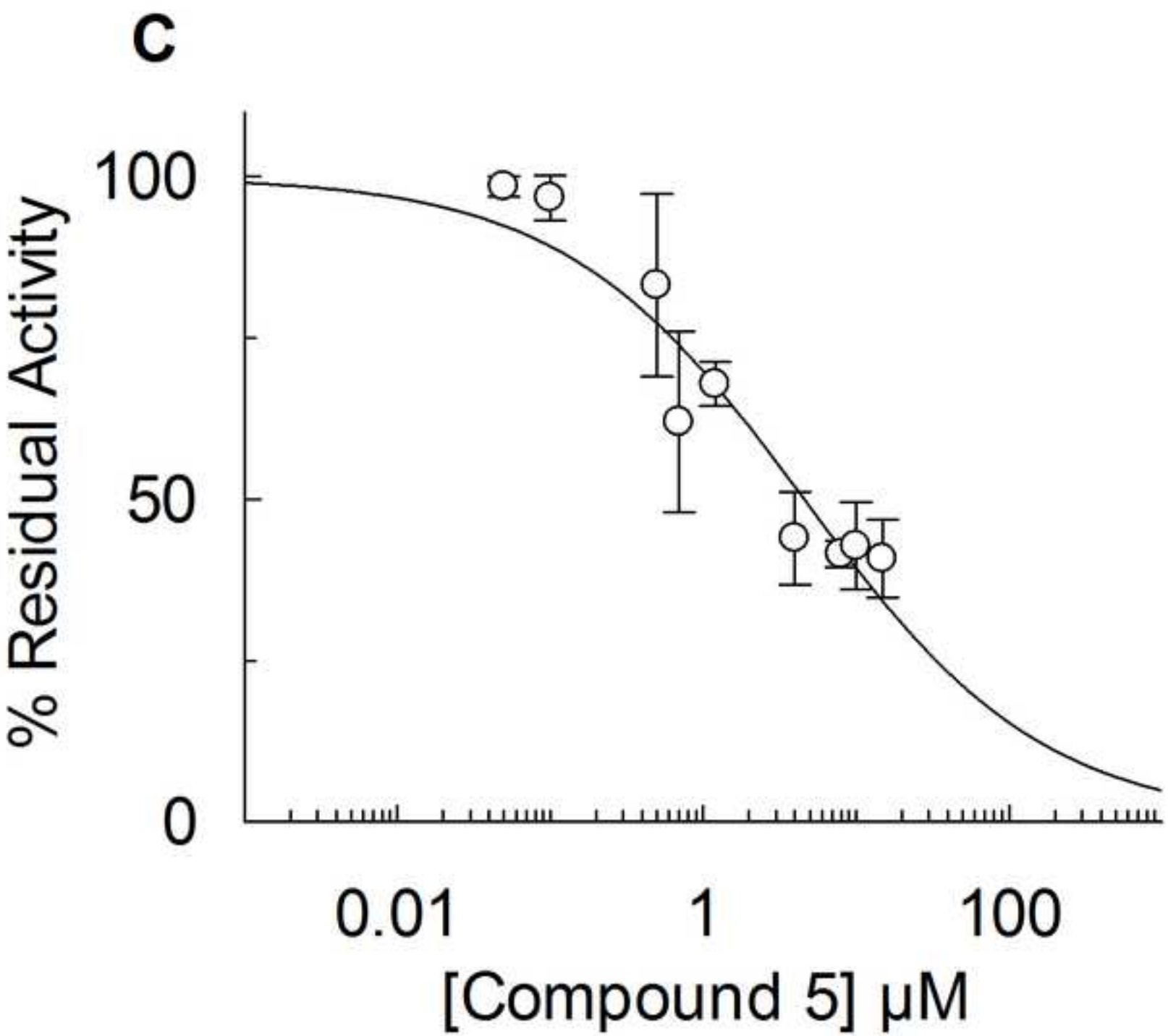

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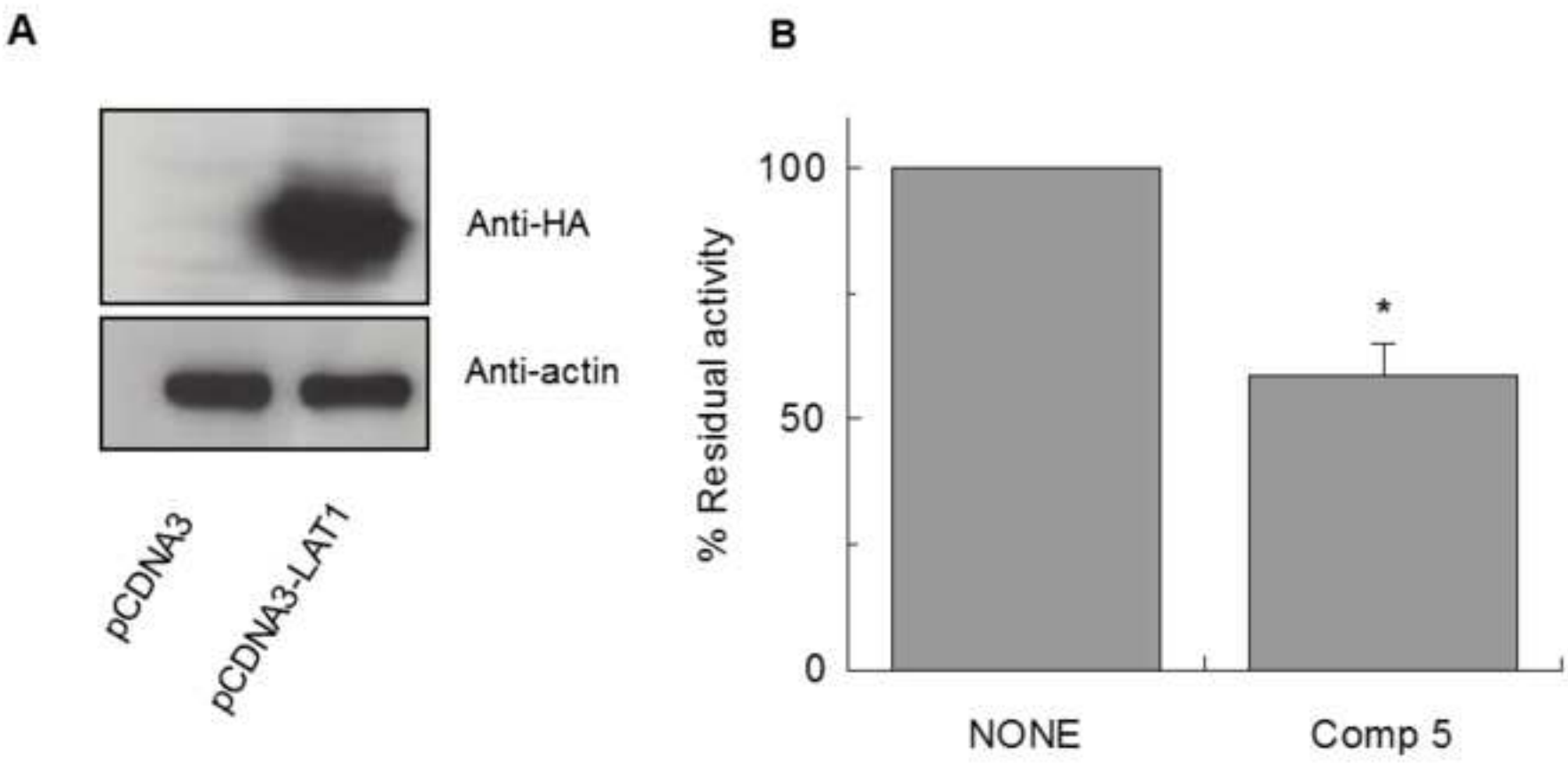

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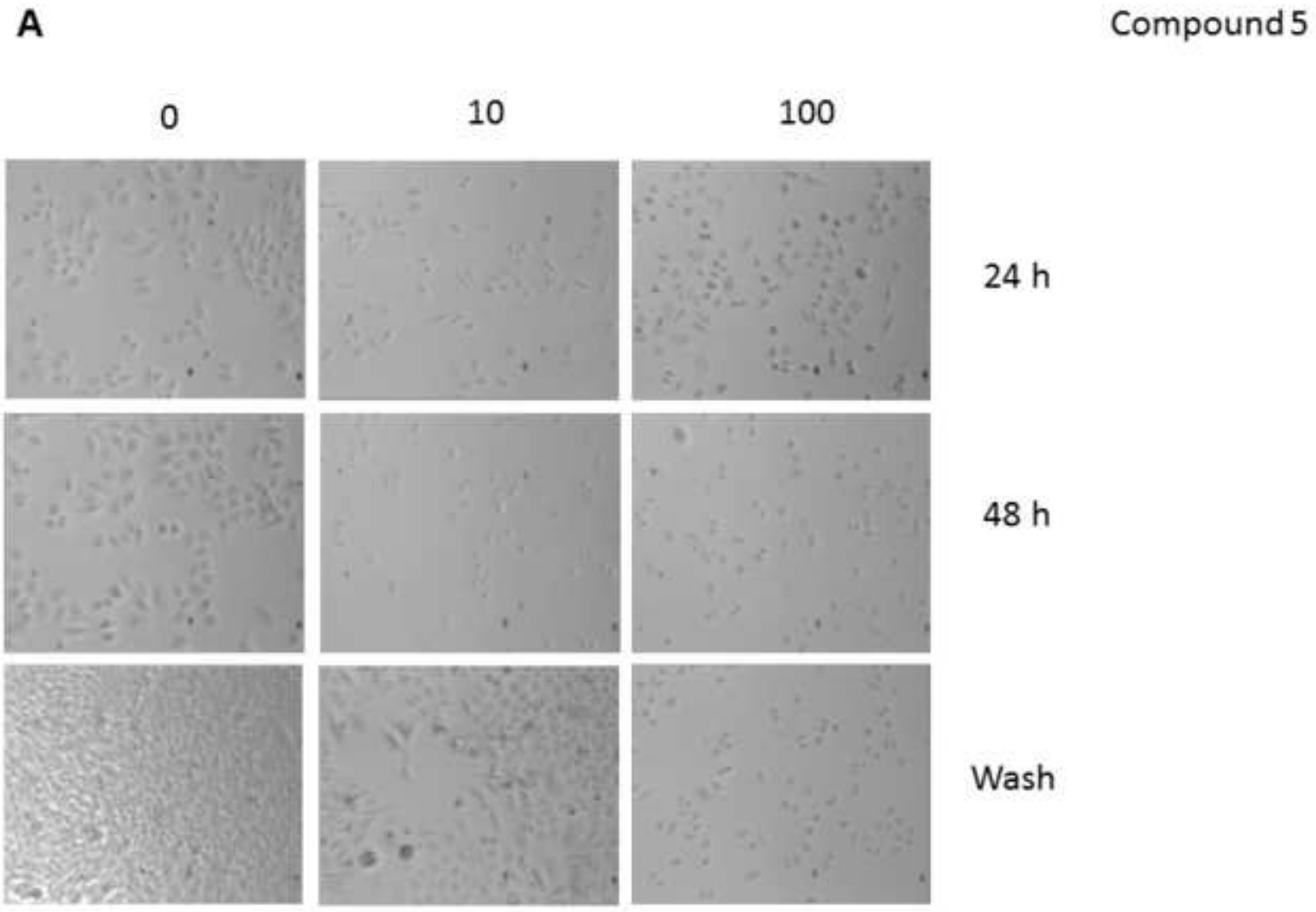

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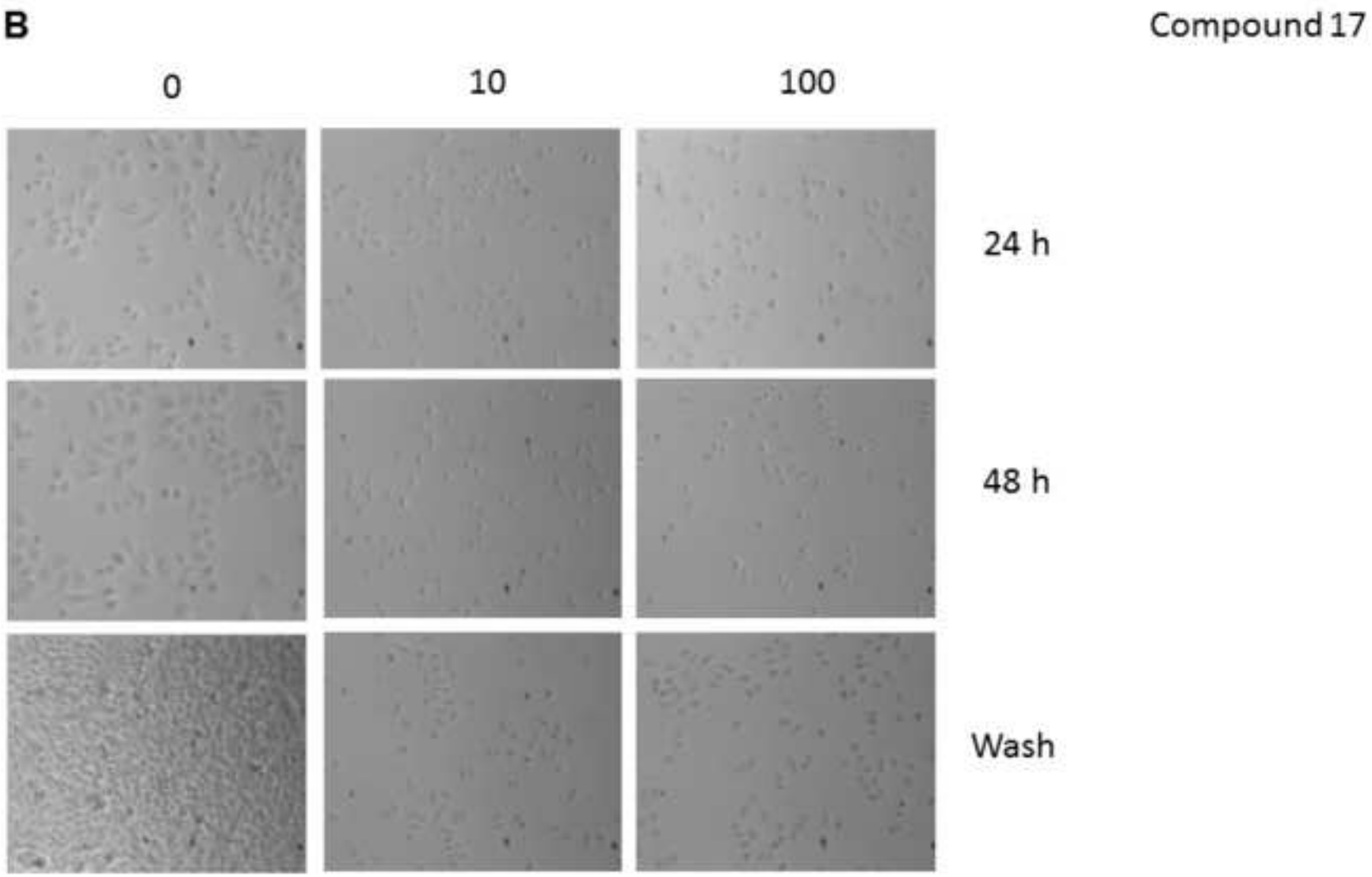

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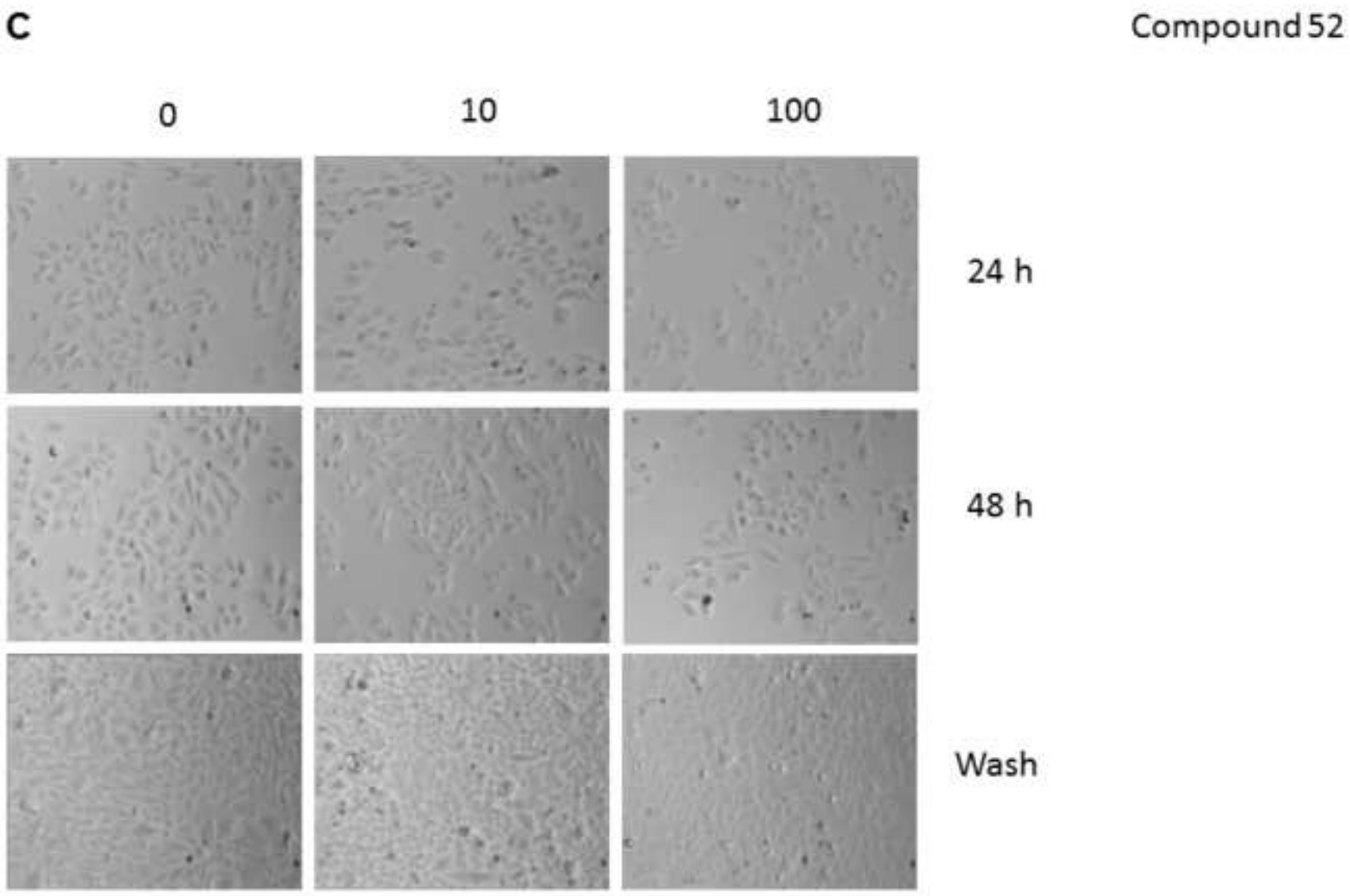

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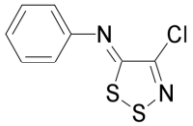
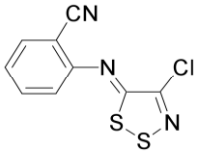
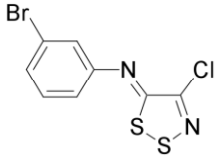
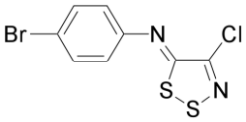
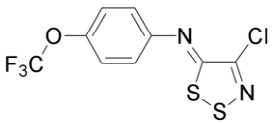
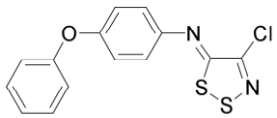
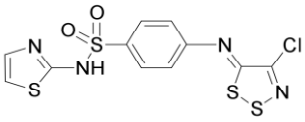
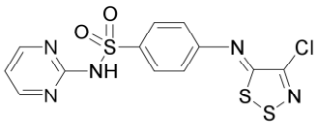
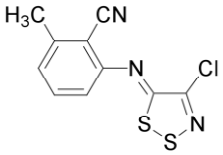
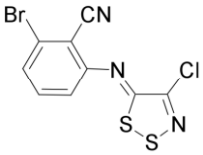
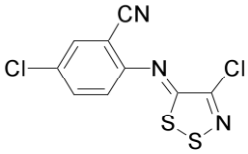
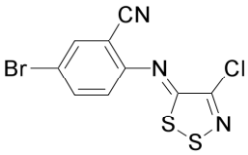
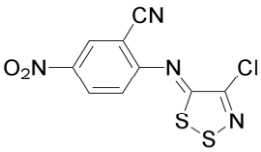
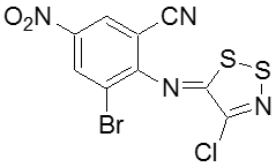
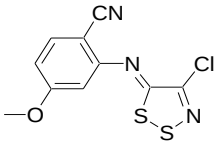
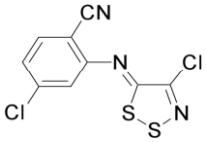
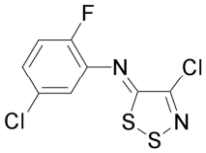
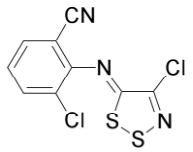
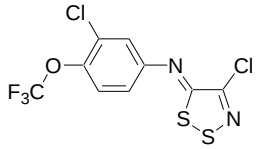
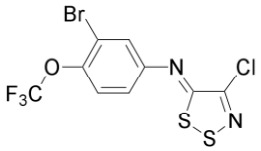
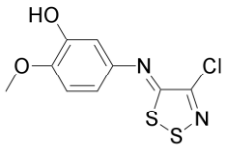
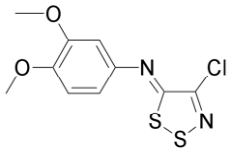
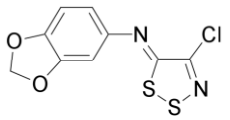
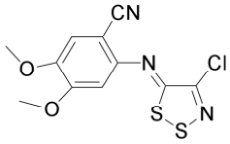
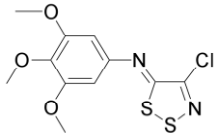
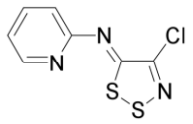
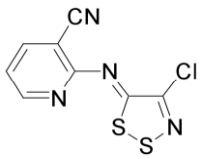
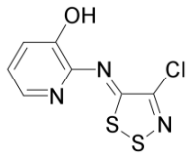
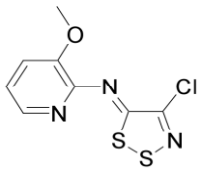
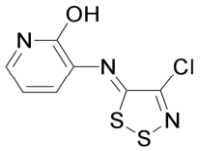
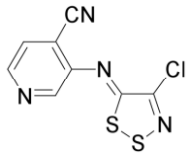
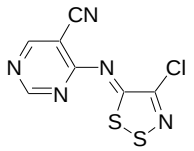
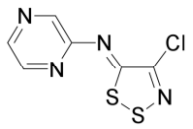
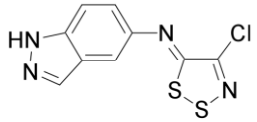
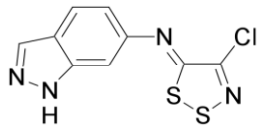
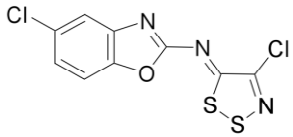
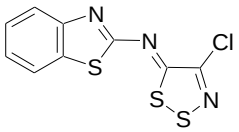
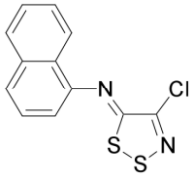
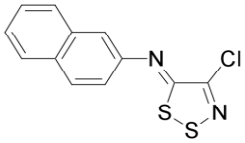
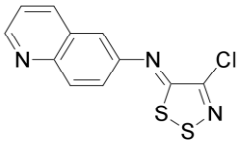
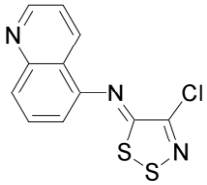
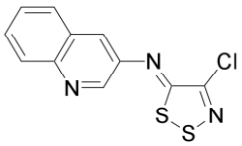
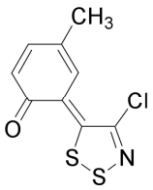
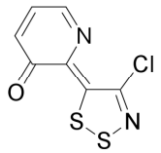
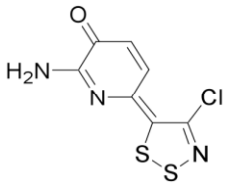
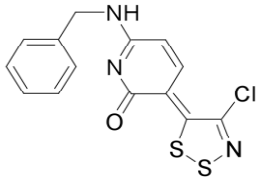
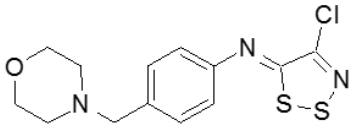
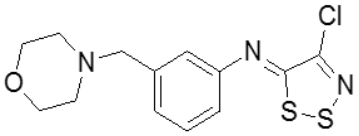
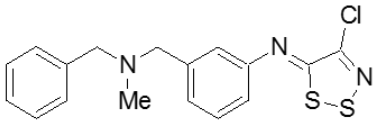
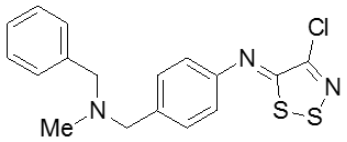
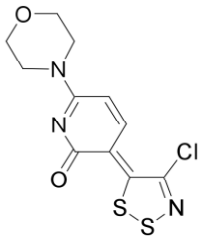
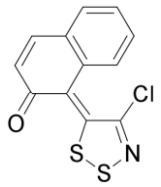
1		81-82
2		44-45
3		82-83
4		68-69
5		> 90
6		62-63
7		63-64

8		81-82
9		63-64
10		> 90
11		> 90
12		> 90
13		88-89
14		85-86
15		85-86
16		> 90
17		> 90

18		78-79
19		> 90
20		68-71
21		31-33
22		67-69
23		49-51
24		83-84
25		65-66

26		4-9
27		41-49
28		35-45
29		10-15
30		67-68
31		87-89
32		75-79
33		63-66

34		29-31
35		48-49
36		66-67
37		67-68
38		59-61
39		58-59
40		74-75
41		65-66
42		84-85
43		70-71

44		48-49
45		63-66
46		66-69
47		61-62
48		58-59
49		51-52
50		48-49
51		26-30
52		72-73

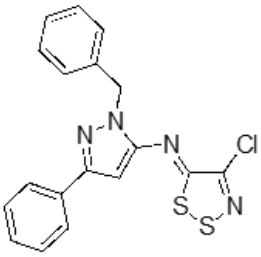
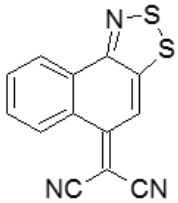
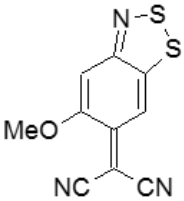
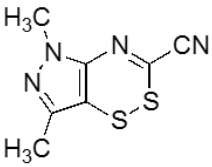
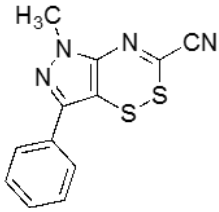
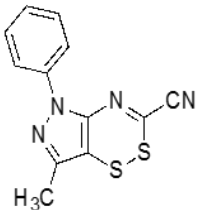
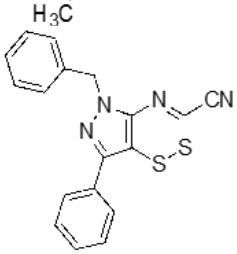
53		48-49
54		60-62
55		0
56		88-89
57		71-72
58		77-78
59		> 90

Table 1

Inhibition of hLAT1 by 1,2,3-dithiazoles **1-55** and 1,2,4-dithiazines **56-59.**
Compounds structures (1-59) prepared as described in Materials and Methods were tested as inhibitors of hLAT1 at 100 µM concentration. Transport activity was assayed in proteoliposomes as [^{3}H]His_{ex}/His_{in} antiport as described in Materials and Methods. Percentage of inhibition from two experiments are reported (see section 2.10). > 90% is reported in case of both values higher than 90%.

Graphical Abstract (for review)
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