SUPPLEMENTARY INFORMATION

DIFFERENTIAL CELLULAR ACCUMULATION MECHANISMS FOR PLATINUM. GLYCOSOAMINOGLYCAN-MEDIATED ENTRY PATHWAY FOR CHARGED TRI-PLATINUM COMPOUNDS.

Heveline Silva[1], Frédéric Frézard[2], Erica J. Peterson[1], Peyman Kabolizadeh[1], John J. Ryan[3] and Nicholas P. Farrell[1*]

[1]Department of Chemistry and [3]Department of Biology, Virginia Commonwealth University, Richmond, Virginia. [2]Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio 6627, 31270-901 Belo Horizonte, MG, Brazil.

Table S1. Dependence of TAMRA-R_9 internalization on GAG status and platinum complexes.[a]

			TAMRA-R9 Fluorescence (%) in:			
Compound	Conc.	CHO-K1	CHO-pgsD677	CHO-pgsA745	HCT116	SAOS-2
Control	-	89.6 ± 8.4	33.9 ± 6.3	3.4 ± 1.5	88.4 ± 4.3	92.12 ± 1.4
TriplatinNC	10 µM	9.9 ± 4.3	-	-	10.7 ± 5.6	17.3 ± 1.1
TriplatinNC	5 µM	16.7 ± 5.9	-	-	18.2 ± 4.1	-
TriplatinNC	1 µM	91.4 ± 4.4	-	-	92.9 ± 2.1	-
AH44	10 µM	86.7 ± 3.4	-	-	68.4 ± 7.5	68.7 ± 0.8
BBR3464	10 µM	88.3 ± 7.9	-	-	77.6 ± 10.7	69.0 ± 6.9
Cisplatin	10 µM	96.2 ± 1.6	-	-	86.7 ± 5.1	88.7 ± 1.3
Oxaliplatin	10 µM	97.0 ± 1.9	-	-	91.3 ± 4.9	92.7 ± 1.8C

a: See Materials and Methods.

Table S2. IC_{50} values of platinum compounds against CHO cells.[a]

Cell Line	TriplatinNC	AH44	BBR3464	Cisplatin
CHO-K1	0.5 ± 0.01	7.63 ± 0.9	2.87 ± 0.05	26.95 ± 0.5
CHOpgs-D677	26.51 ± 0.9	> 50	> 50	28.02 ± 1.3
CHOpgs-A745	14.21 ± 3.1	> 50	9.71 ± 1.0	33.21 ± 0.6

a: IC_{50} is concentration required to inhibit 50% growth. Values are mean of triplicate repetition $\pm$ stanadard deviation.

Figure legends.

Figure S1. TAMRA-R_9 internalization into living CHO cells is glycosaminoglycan dependent. The internalization of TAMRA-R_9 (1.0 μM) was analyzed by fluorescence microscopy. Cells were counterstained with Hoescht 33342. Panels: (A) CHO-K1 cells (wild-type); (B) CHO-pgsD-677 cells (which lack HS); (C) CHO-pgsA-745 cells (which lack HS and CS). (1) Bright-field image (2) Fluorescence image. Bars = 10 μm.

Figure S2. TAMRA-R_9 Fluorescence percentage by flow cytometry. Addition of TAMRA-R_9 (1.0 μM) and analyzed by flow cytometry after 60 min incubation and six times washed with PBS. (A) TAMRA-R_9 uptake in CHO wild-type and GAG deficient cells. Statistics analysis used was One Way ANOVA and Bonferoni post-test, all bars no signalized are significant different ($p < 0.01$). Each point is the average (+/- SD) of three independent experiments.

Figure S3. Electrospray Ionization Mass Spectrum of octasaccharide DP8 (structure, top left) in presence of TriplatinNC. The major peak corresponds to a 1:1 adduct and the presence of the trinuclear unit (See Fig. 1) is confirmed by isotopic distribution (red). In this case the major peak corresponds to the 3- state of a 1:1 adduct with loss of 3 SO_3^- and addition of 3 Na^+ {See Saad OM, Leary JA. (2003) Compositional analysis and quantification of heparin and heparan sulfate by electrospray ionization ion trap mass spectrometry. *Anal. Chem*. 75:2985-2995}.

Figure S4. Influence of GAGs in apoptosis assay. Apoptosis induction by TriplatinNC and cCisplatin (CDDP) and oxaliplatin was measured using Chinese Hamster Ovary cells lines (CHO-K1; CHO-pgsD-677, lack HS; and CHO-pgsA-745, lack HS and CS). CHO cells were cultured in the indicated concentrations of platinum drugs for 72h, fixed and stained with propidium iodide (PI) and RNAse A. Percent apoptosis induction was determined by comparing the intact versus fragmented DNA of cells from treated and untreated cultures after 72h, as measured by PI-DNA staining and timed counting via flow cytometry as described in Materials and Methods. Each point is the average (+/- SD) of three independent experiments.

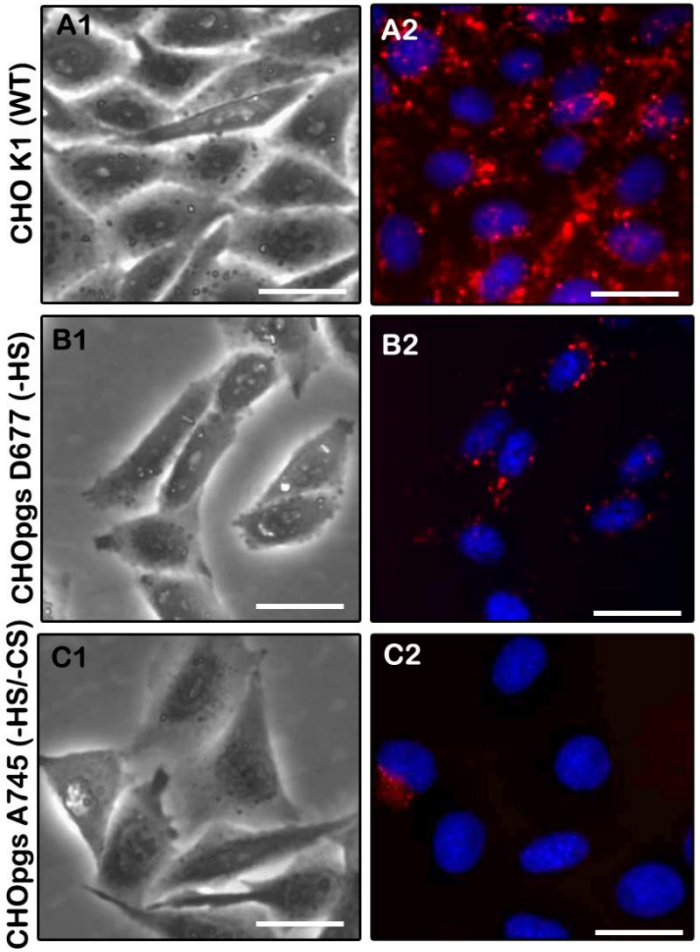
Figure S5. Effect of the macropinocytosis inhibitors cytochalasin D and EIPA on the uptake of the trinuclear platinum drugs BBR3464 and TriplatinNC by HCT116 cells. A,B: cells were pre-treated 30 min in complete RPMI medium containing or not 20 μM cytochalasin D and incubation in the presence of drug was continued for 4 h (A, 40 μM BBR3464, B, 10 μM TriplatinNC). C,D: Cells were pre-treated 30 min in hepes saline buffer containing or not 0.1 mM EIPA. The drug was then added and cells were incubated for 1 h (C, 60 μM BBR3464, D, 40 μM TriplatinNC). Data are shown as mean ± SD (n=3). * p = 0.05, ** p <0.01, Student t test.

Figure S6. Effect of Nystatin, inhibitor of lipid-raft macropinocytosis, on the accumulation of the trinuclear platinum drugs BBR3464 (A) and TriplatinNC (B) by HCT116 cells. Cells were pre-treated 30 min in Hepes saline buffer containing or not 50 μg/ml nystatin and then incubated for 1 h in the presence of the drug (60 μM BBR3464, 40 μM TriplatinNC); Data are shown as mean ± SD (n=3). *p = 0.05, ** p<0.01, Student t test.

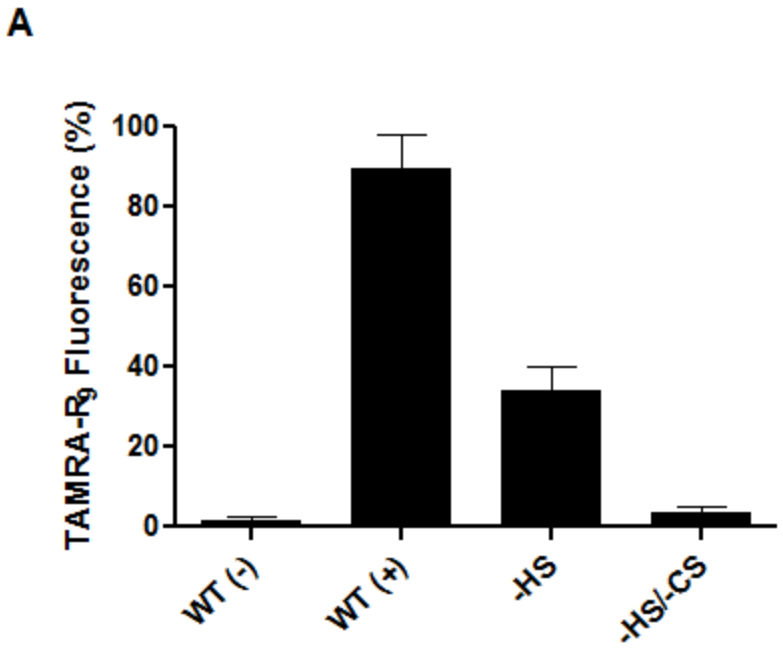
Figure S7. Effect of hypertonic Hepes-NaCl-sacarose buffer (A, B) and chlorpromazine (C, D), inhibitors of clathrin-mediated endocytosis, on the uptake of the trinuclear platinum drugs BBR3464 (A, C) and TriplatinNC (B, D) by HCT116 cells. A, B: Cells were incubated for 90 min in Hepes saline buffer containing or not 0.43 M sucrose in the presence of the drug (60 µM BBR3464, 40 µM TriplatinNC). C, D: Cells were pretreated for 30 min in isotonic Hepes saline buffer containing or not 30 µM chlorpromazine and then incubated for 1 h in the presence of the drug (60 µM BBR3464, 40 µM TriplatinNC). Data are shown as mean ± SD (n=3). *p<0.05, Student t test.

Figure S8. The effect of cimetidine on Pt-compounds cellular accumulation**.** In A, B, C, HCT116 cells were treated with 20µmol/L BBR3464 for 8h, and c-DDP, Oxaliplatin for 16h, and cellular platinum content was measured by ICP-OES. Each point represents the average (+/- SEM) of three independent experiments. *, p<0.05 as determined by t test. In D, Triplatin NC (20µM) was added 1h after Cimetidine. Time of drug exposure 8h. No significant difference was found. (One-Way ANOVA, Bonferroni Post-test)

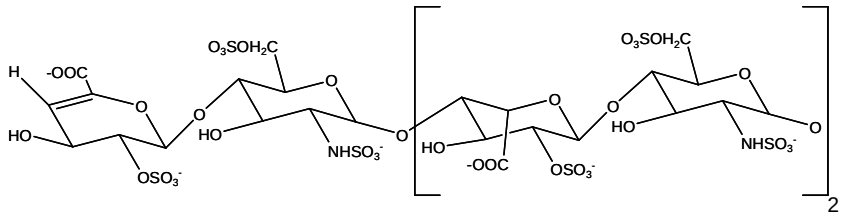
S1

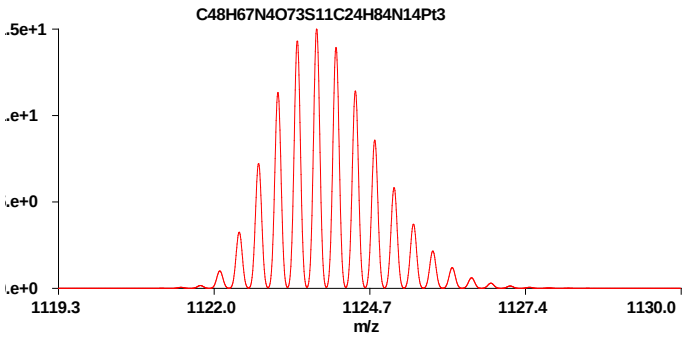


S2



S3

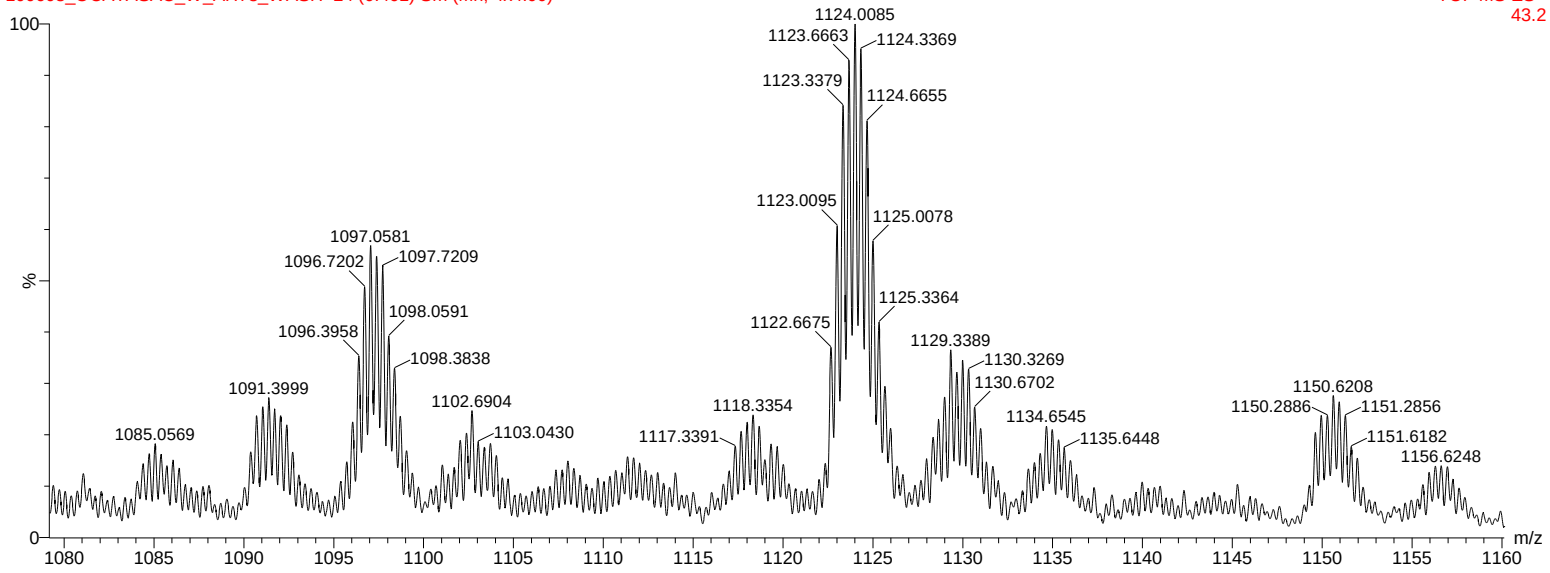




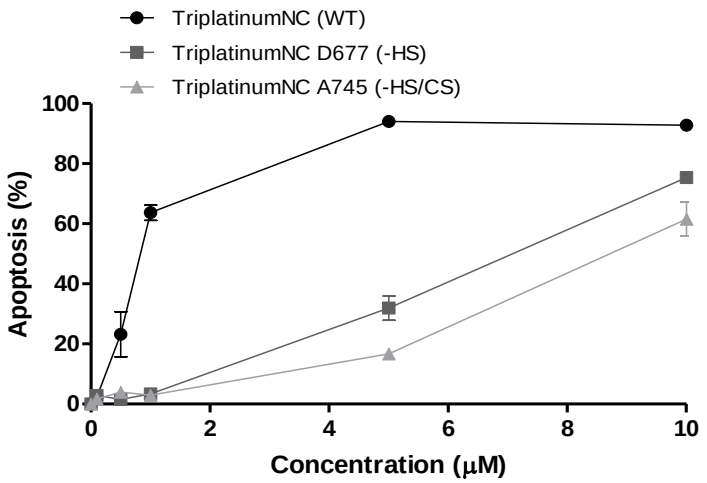
neg ion negion buffer mw 2664(Na16)

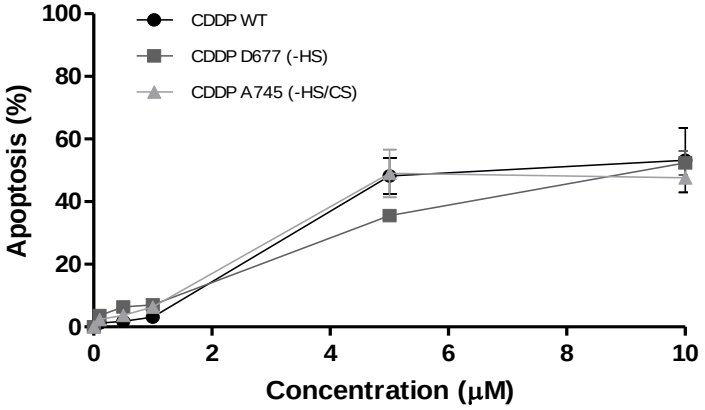
100608_OCATASAC_W_AH78_WASH 24 (0.461) Sm (Mn, 4x4.00)

TOF MS ES-
43.2

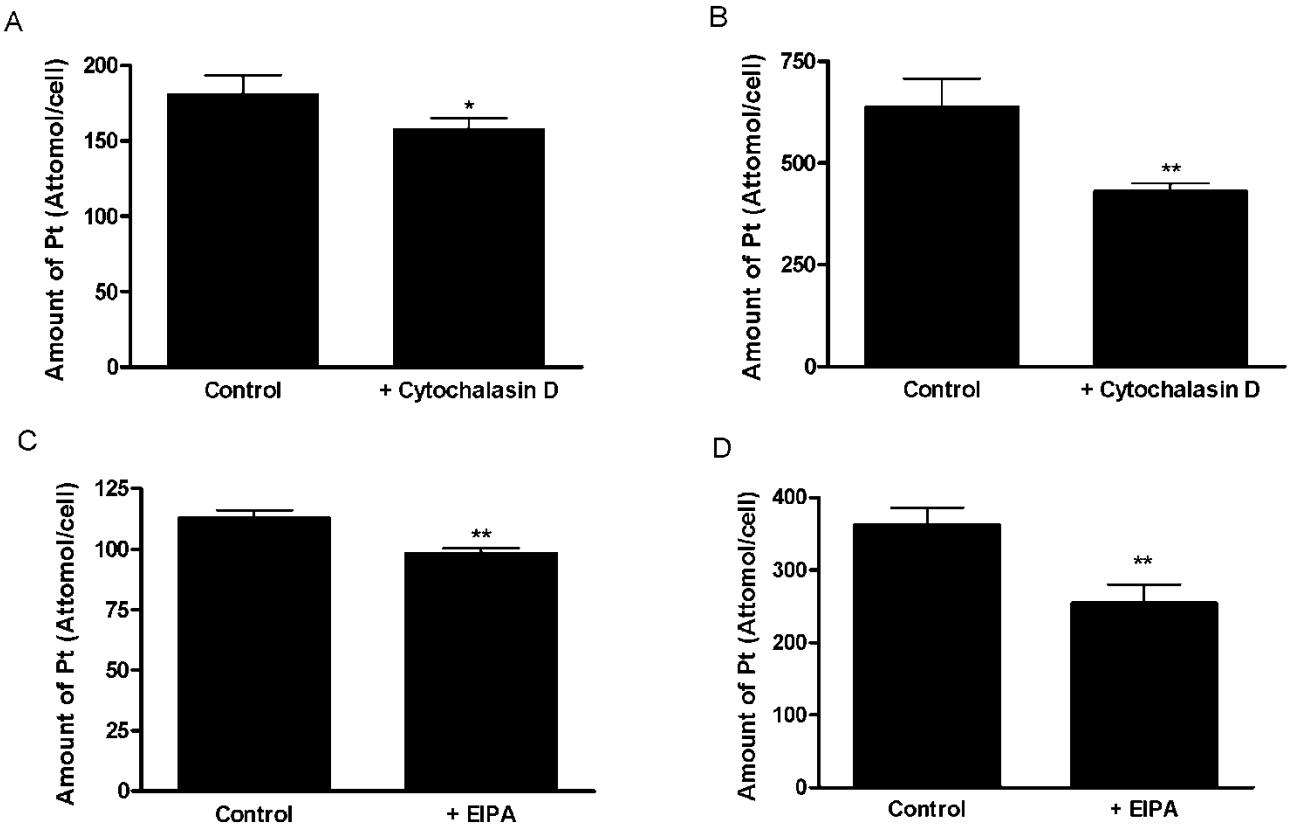


S4

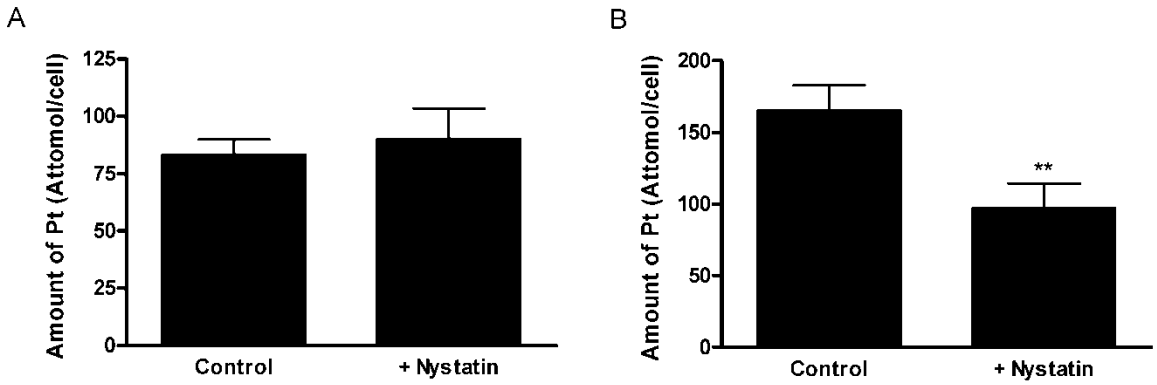

TriplatinumNC (WT)
TriplatinumNC D677 (-HS)
TriplatinumNC A745 (-HS/CS)
Apoptosis (%)
100
80
60
40
20
0
0
2
4
6
8
10
Concentration (μM)


CDDP WT
CDDP D677 (-HS)
CDDP A745 (-HS/CS)
Apoptosis (%)
100
80
60
40
20
0
0
2
4
6
8
10
Concentration (μM)

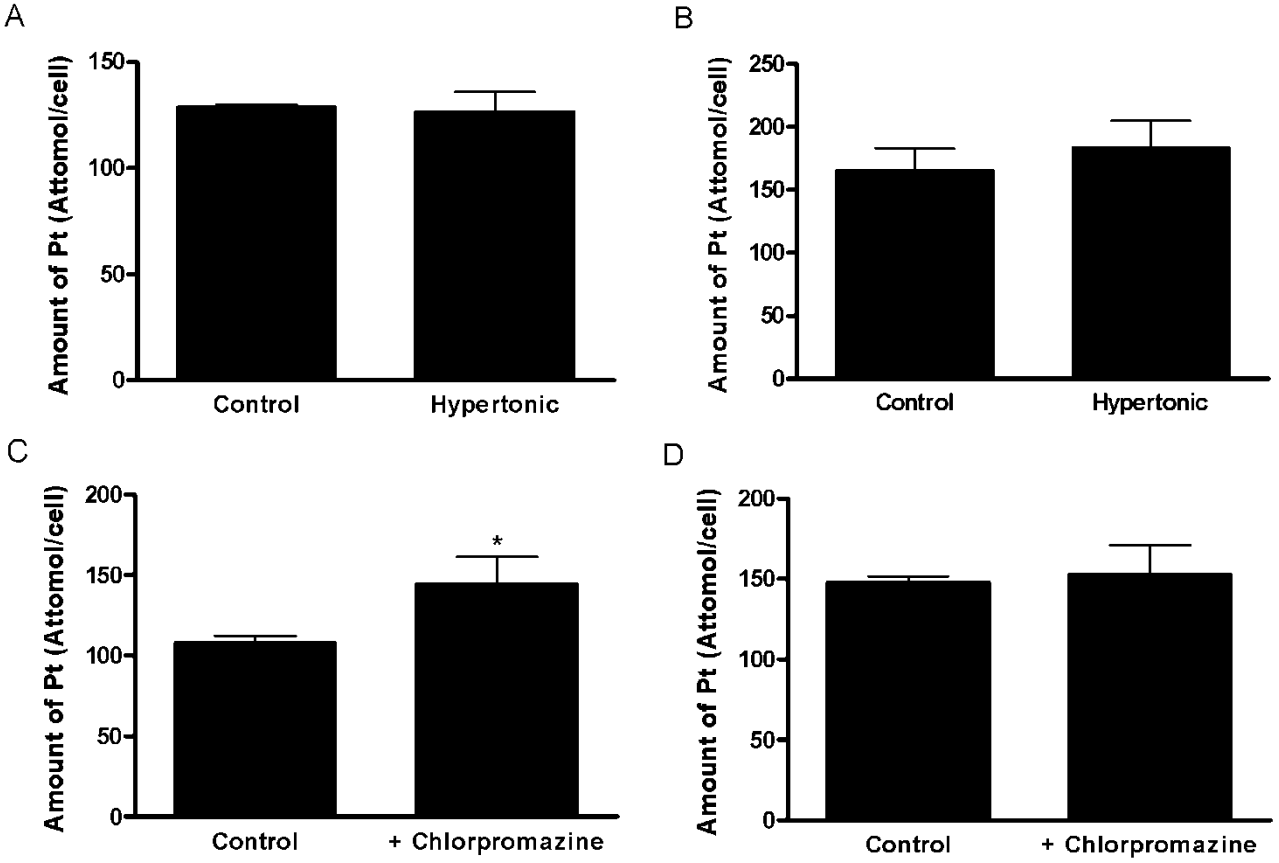
S5


A
Amount of Pt (Attomol/cell)
200
150
100
50
0
*
Control
+ Cytochalasin D
B
Amount of Pt (Attomol/cell)
750
500
250
0
**
Control
+ Cytochalasin D
C
Amount of Pt (Attomol/cell)
125
100
75
50
25
0
**
Control
+ EIPA
D
Amount of Pt (Attomol/cell)
400
300
200
100
0
**
Control
+ EIPA

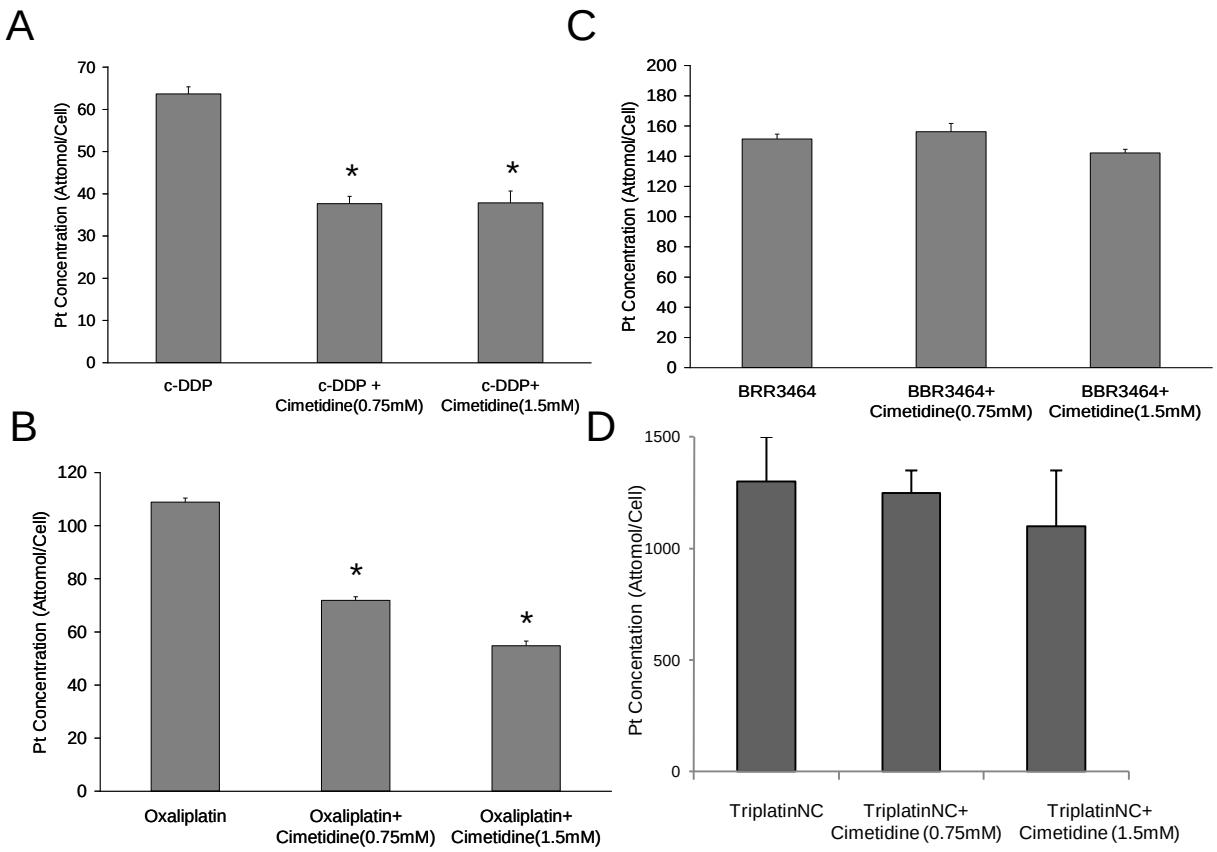
S6


A
Amount of Pt (Attomol/cell)
125
100
75
50
25
0
Control
+ Nystatin
B
Amount of Pt (Attomol/cell)
200
150
100
50
0
**
Control
+ Nystatin

S7


A
Amount of Pt (Attomol/cell)
150
100
50
0
Control
Hypertonic
B
Amount of Pt (Attomol/cell)
250
200
150
100
50
0
Control
Hypertonic
C
Amount of Pt (Attomol/cell)
200
150
100
50
0
*
Control
+ Chlorpromazine
D
Amount of Pt (Attomol/cell)
200
150
100
50
0
Control
+ Chlorpromazine

S8


A
Pt Concentration (Attomol/Cell)
70
60
50
40
30
20
10
0
*
*
c-DDP
c-DDP + Cimetidine(0.75mM)
c-DDP+ Cimetidine(1.5mM)
B
Pt Concentration (Attomol/Cell)
120
100
80
60
40
20
0
*
*
Oxaliplatin
Oxaliplatin+ Cimetidine(0.75mM)
Oxaliplatin+ Cimetidine(1.5mM)
C
Pt Concentration (Attomol/Cell)
200
180
160
140
120
100
80
60
40
20
0
BRR3464
BBR3464+ Cimetidine(0.75mM)
BBR3464+ Cimetidine(1.5mM)
D
Pt Concentation (Attomol/Cell)
1500
1000
500
0
TriplatinNC
TriplatinNC+ Cimetidine (0.75mM)
TriplatinNC+ Cimetidine (1.5mM)